Improvements in

Cataract Surgery

Lisanne Maria Nibourg

Improvements in Cataract Surgery

Lisanne Maria Nibourg

Colofon

Ontwerp kaft en binnenwerk:	Esther Ris, Proefschriftomslag.nl, Koog aan de Zaan
Druk:	Ridderprint BV, Ridderkerk
ISBN (gedrukte versie):	978-90-367-8538-9
ISBN (elektronische versie):	978-90-367-8537-2

The printing of this thesis was kindly supported by the University of Groningen, University Medical Center Groningen, Professor Mulder Stichting, and Abbott Medical Optics Groningen.

rijksuniversiteit
groningen

Improvements in Cataract Surgery

Proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. E. Sterken
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 27 januari om 12.45 uur

door

Lisanne Maria Nibourg

geboren op 29 januari 1987
te Hengelo

Promotor:	Prof. dr. J.M.M. Hooymans
Copromotores:	Dr. S.A. Koopmans Dr. T.G. van Kooten
Beoordelingscommissie:	Prof. dr. G.P.M. Luyten Prof. dr. R.M.M.A. Nuijts Prof. dr. Y. Ren

Paranimfen:	S.A.F. Nibourg
	B. Wullink

Table of contents

Part 2: Improvement of techniques in cataract surgery

Chapter 1

Introduction and aims of this thesis

1.1 Cataract

Cataract is the leading cause of blindness and visual impairment in the elderly all through the world (Klein and Klein, 2013).[A] It involves opacification of the eye lens due to biochemical changes in the lens cells and the extracellular matrix surrounding the cells (Lovicu et al., 2015). The name cataract originates from the Latin word *cataracta* meaning waterfall, as a cataractous lens may resemble the gray colors which can be seen in large waterfalls (Figure 1). The normal lens consists of a nucleus and a cortex, consisting of crystalline lens fibers, surrounded by a lens capsule (Figure 2). On the basis of these lens components, three types of cataract can be distinguished: nuclear, cortical, and subcapsular cataracts. Mainly combinations of these types are present in patients (Klein and Klein, 2013). Cataract results in a decrease in visual acuity due to different causes, depending on the type of cataract: e.g. lens opacification, light scattering, or changes in the refractive index of the lens. The treatment for all types of cataract is removal of the opaque lens by cataract surgery followed by implantation of an artificial lens.

FIGURE 1. *Photo of a lens from a patient with a gray mature cataract.*

Accommodation and presbyopia

In an emmetropic eye, together with the optical power of the cornea, the lens focuses light originating from distant objects into an image which is projected on the retina (Figure 3). For viewing of objects closer to the eye, the anterior curvature of the lens decreases which changes the focal distance of the eye. This change in lens shape is a result of contraction of the ciliary muscle. This muscle surrounds the lens and the lens is suspended from the ciliary muscle through the zonular fibers via the ciliary processes. The change in optical power of the eye resulting from contraction of the ciliary muscle is called accommodation.

Aging is accompanied by stiffening of the lens and results in a decrease of the accommodative amplitude of the eye (Fisher, 1988, Strenk et al., 1999, Weeber and van der Heijde, 2007). At the age of 40 to 50 years, reading may become difficult due to the inability to obtain a well focused image of nearby objects. This is called presbyopia and most people will need reading glasses which compensate for the inability to obtain a focused image of nearby objects. It has long been thought that presbyopia was caused by a combination of lens stiffening and a decrease of the contractive force of the ciliary muscle (Fisher, 1988). However, it was found that the ciliary muscle still contracts in presbyopic eyes, so the main cause of presbyopia seems to be the stiffening of the lens itself (Strenk et al., 1999).

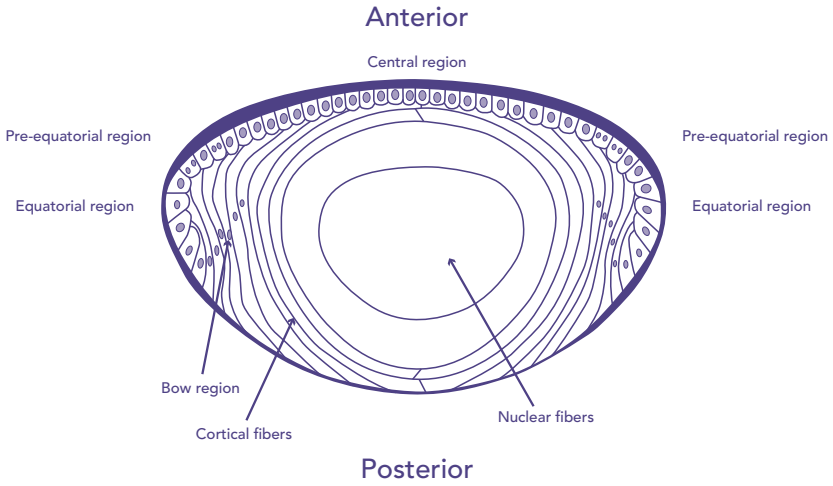


FIGURE 2. *Schematic cross-section of the lens, showing different regions and cells which are present in the normal human lens.*

Cataract surgery

A modern cataract operation provides restoration of vision by removal of the opaque lens nucleus and cortex, followed by implantation of an intraocular lens (IOL) in the lens capsule. During this surgery, the lens nucleus and cortex are extracted through an opening in the anterior part of the lens capsule (curvilinear continuous capsulorhexis, CCC), but the main part of the capsule remains in the eye in order to secure the implanted IOL (Pande et al., 1996a, Spalton et al., 2014).

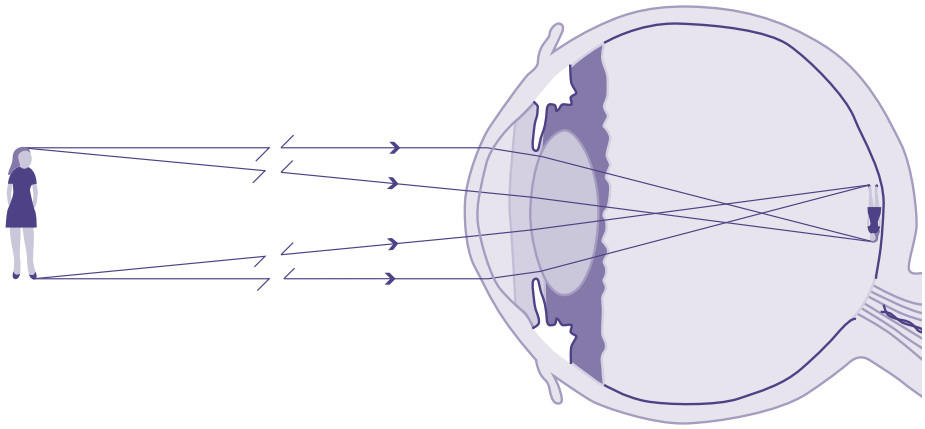


FIGURE 3. *Schematic overview of the projection of an image from a distant object on the retina. The cornea and lens focus the light beams into the focal point on the retina.*

Improvement of intraocular lenses for cataract surgery

Intraocular lenses have been improved throughout the years since the first IOL was implanted by Sir Harold Ridley in 1949 (Apple et al., 1992, Dewey et al., 2014, Moore et al., 2010, Nibourg et al., 2015b, Ong et al., 2014, Spalton, 2009). Modern IOLs are foldable and therefore can be implanted in the eye through small incisions, which minimizes tissue trauma to the eye. The IOLs are available in many different optical powers so that existing sphero-cylindrical refractive errors in the eye of the patient can be corrected. The choice of the IOL optical power is determined by precise preoperative measurement of the dimensions of the eye.

Nowadays, the majority of the implanted IOLs are monofocal. This means that the image of an object at one distance is focused on the retina. Usually, the power of the implanted IOL is chosen so that emmetropia is achieved. This means that the patient will see distant objects clearly, but will need reading glasses to see clearly at an intermediate and nearby distance. In order to have a well focused image of objects at various distances from the eye, the intraocular lens would have to be able to vary its optical power, similar to the natural crystalline lens. When such an accommodative intraocular lens is implanted, rendering the patient emmetropic, the patient would not need reading glasses anymore. In recent years, various types of accommodative intraocular lenses have been designed and used in preclinical and clinical studies (Glasser, 2008, Nishi et al., 2009, Ong et al., 2014).

A promising type of such an IOL is the injectable accommodative lens. An injectable accommodative lens is implanted after removal of the lens nucleus and cortex through a small opening in the anterior lens capsule, followed by injection of a soft transparent polymer which refills the lens capsular bag (Haefliger et al., 1987, Koopmans et al., 2003, Koopmans et al., 2006, Nibourg et al., 2015a, Nishi and Nishi, 1998, Nishi et al., 2009). The goals of this type of surgery and lens implantation are to provide clear vision by removal of the opacified lens and to restore accommodation to the presbyopic patient (Figure 4). Preclinical experiments using the accommodative lens refilling technique have been carried out in *ex vivo* models and in *in vivo* experiments using rabbits and rhesus monkeys (Nishi et al., 2009). Lens refilling in rhesus monkeys showed that accommodation can be maintained using this technique (Koopmans et al., 2006). However, after implantation capsular opacification developed which caused a decrease of the accommodative amplitude and a decreased clarity of the lens (Koopmans et al., 2011, Nibourg et al., 2015a, van Kooten et al., 2006). Capsular opacification is also an important issue in other types of accommodative lenses, where it can cause stiffening of the lens capsule and it reduces the accommodative amplitude. Therefore, prevention of capsular opacification is essential in accommodative lenses.

A B

FIGURE 4. *Pictures of a porcine lens which is refilled with a silicone polymer before (A) and after (B) extraction from the eye. The capsular bag is closed with a transparent plug, consisting of two layers and positioned outside the visual axis. The extracted lens (B) is presented on Westcott scissors.*

1.2 Capsular opacification

Capsular opacification (CO) is also named secondary cataract and is a common complication of cataract surgery (Wormstone et al., 2009). CO is a consequence of growth and abnormal proliferation of lens epithelial cells (LECs) after cataract surgery. LECs are situated in a single layer on the inner side of the anterior lens capsule, the pre-equatorial, and equatorial regions of the lens (Figure 2) (Apple et al., 1992, Awasthi et al., 2009, Marcantonio and Vrensen, 1999). After cataract surgery, remaining LECs migrate over the posterior lens capsule and grow into the visual axis resulting in a diminished visual acuity several months to years after the initial surgery. In modern cataract surgery most of the anterior capsule is removed by the CCC, leaving only the posterior capsule for outgrowth of LECs into the visual axis. That is why this process is widely referred as posterior capsular opacification (PCO). However, throughout this thesis the term capsular opacification will be used because for new accommodative lens designs the anterior capsule as well as the posterior capsule may remain in the eye and both may become opacified causing visual disturbances due to LEC growth and decrease of the accommodative amplitude due to stiffening.

Pathogenesis of capsular opacification

The pathogenesis of CO and the underlying biological pathways are discussed in detail in Chapter 2. In short, the development of CO is based on an inflammatory response which results in a combination of three processes in the LECs: proliferation, migration, and transdifferentiation (Lopez-Novoa and Nieto, 2009, Pande et al., 1996b). Proliferation means an increase in the number of cells due to cell growth and division. Migration entails the movement of cells towards the visual axis. This becomes possible due to different cell attachment molecules which enable the cells to move over the lens capsule (Nishi et al., 1997, Zhang et al., 2000). Furthermore, LECs in the equator may change from cell type and can transdifferentiate into cells which resemble the normal crystalline lens fibers (Figure 2). LECs on the anterior side of the lens capsule can undergo transformation to myofibroblasts (de Iongh et al., 2005). This transformation is an expression of the biological process of epithelial to mesenchymal transformation (EMT) (Nieto, 2011). As a result of this process, the lens capsule surface may become wrinkled. When this occurs in the visual axis, it causes a decrease in clarity of the lens capsule resulting in a decrease in visual acuity (Marcantonio et al., 2003).

Process of epithelial to mesenchymal transformation

EMT is a biological process that can occur in many tissues and entails the conversion of epithelial cells into mesenchymal cells. Epithelial cells grow in ordered layers and form the lining between tissues or between a tissue and a cavity. Mesenchymal cells have migratory and invasive properties allowing these cells to move and grow into other tissues. The process of EMT takes place in various processes of the human body, such as the formation of organs by stem cells, regeneration of tissues and wound healing, and in diseases like fibrosis and cancer (Lopez-Novoa and Nieto, 2009). For example, the process of EMT is activated during the progression of cancer and is an important step in the metastatic cascade of epithelial tumors. In the lens, EMT is currently associated with two processes: the development of anterior subcapsular cataract and the formation of CO (de Iongh et al., 2005, Eldred et al., 2011). The process of EMT has long been recognized, but now that specific steps of the process became known this knowledge can be used for the development of new therapies in diseases like fibrosis and cancer.

Current treatment of capsular opacification

When CO causes a decrease in visual acuity it is generally treated with a Nd:YAG laser capsulotomy (Aslam et al., 2003). This laser is used to create an opening in the center of the posterior capsule in order to provide clear vision for the patient. The therapy is widely used but not available for all patients suffering from CO, since the Nd:YAG laser device is expensive. Furthermore, side effects to other ocular tissues are described after Nd:YAG laser capsulotomy, including elevation of the intraocular pressure, damage to the IOL, macular edema, and an increased risk of retinal detachment (Aslam et al., 2003, Ge et al., 2000, Steinert et al., 1991). This makes that there is a need for the prevention of CO.

Developments in the prevention of capsular opacification

Prevention of CO is a major area of interest in the field of cataract surgery. Many changes to IOL materials and surgical techniques have been developed aiming to decrease CO formation (Apple et al., 1992, Findl et al., 2010, Hazra et al., 2012). Methods involve e.g. different designs and materials of IOLs providing a decreased inflammatory response or a mechanical barrier to prevent migration of LECs (Hazra et al., 2012, Nishi et al., 2000). Furthermore, different surgical techniques to remove LECs or methods for performing the capsulorhexis have been tried (Birinci et al., 1999, Vasavada et al., 2006). Although partly successful, none of these methods succeeded in a total prevention of CO. The

only surgical method that seems to totally prevent CO is removal of the posterior lens capsule by performing a CCC of the posterior lens capsule (Stifter et al., 2007). This prevents migration of LECs into the posterior visual axis. However, the technique is not widely used, probably because there is an increased risk of vitreous loss and IOL luxation (Georgopoulos et al., 2003, Menapace, 2008).

Prevention of CO is a requirement for the development of new techniques like accommodative lens refilling, since these lenses need an intact and clear lens capsule as a surrounding for the injected polymer in order to provide accommodation of the new refilled lens (Nibourg et al., 2015a, van Kooten et al., 2006). Recent evidence suggests that biomaterials have a potential for influencing the cellular response in tissues (Cui et al., 2010). Therefore, we hypothesized that a suitable biomaterial might influence the response of LECs after cataract surgery. Nanomaterials in the form of nanofiber-based hydrogels (nanogels) were chosen as a possible candidate because they may prevent CO formation by establishment of a layer between the inserted lens refilling material and the surface of the capsular bag (Etheridge et al., 2013, Goldberg et al., 2007, Nibourg et al., 2015b).

1.3 Nanomaterials

Nanomedicine is a relatively new field in medicine and gains an increasing share in biomedical research and applications, also in the field of ophthalmology (Etheridge et al., 2013, Zarbin et al., 2010). Nanomaterials are structures with sizes between the 1 and 1000 nm (Kreyling et al., 2010). The use of these materials in medical applications is increasingly important as they provide new possibilities for interference with biological processes on a cellular level (Etheridge et al., 2013, Goldberg et al., 2007). The structure of nanomaterials can range from spheres, fibers, capsules, tubes, dendrimers, micelles to irregularly shaped particles (Euliss et al., 2006, Gentleman and Chan, 2009) (see Figure 5 in Chapter 2). Nanomaterials can e.g. be used for tissue engineering or drug delivery (Goldberg et al., 2007). In ophthalmology, nanomaterials have been tested for development of artificial corneas and retinal implants (Ionescu et al., 2011, Pritchard et al., 2010), for intraocular drug delivery (Guha et al., 2013, Huang et al., 2013, Kaiser et al., 2013, Li et al., 2013), for stem cell therapy for ocular surface injuries (Ellis-Behnke and Jonas, 2011), and also for the prevention of CO (Guha et al., 2013, Huang et al., 2013, Wang et al., 2013, Zhang et al., 2013).

1.4 Training for cataract surgery

For proper performance of cataract surgery and the implantation of artificial lenses, it is highly important to provide sufficient training to new surgeons (Ament and Henderson, 2011, Binenbaum and Volpe, 2006). Training in cataract surgery usually involves a combination of exercises on a cataract surgery simulator and practice with the surgical microscope in a wet laboratory (wet lab) on model eyes or animal eyes (Henderson et al., 2009, McCannel et al., 2013). Animal eyes may also be used in a wet lab setting by experienced surgeons to test new experimental surgical techniques. Porcine eyes are often used and they can be obtained fresh from the local slaughterhouse (Hashimoto et al., 2001, Koopmans et al., 2004, van Vreeswijk and Pameyer, 1998). However, shortly after the harvesting of these eyes, post-mortem changes start to occur causing a decrease in the clarity of the cornea. Therefore, it is important to know how porcine eyes are best stored so that the optical quality of the cornea is maintained as long as possible.

In cataract surgery, surgical microscopes are necessary to provide a good view of the surgical field. Current surgical microscopes are equipped with stereoscopic binoculars which enable depth perception (Hoerenz, 1980). However, this is only an advantage for surgeons with sufficient stereovision. Therefore, it is important to know if stereovision is necessary for training and properly performing cataract surgery.

1.5 Aims and outline of this thesis

The main objective of this thesis is to examine methods for the improvement of cataract surgery.

Two primary aims were selected:

1. To investigate the effect of different materials for accommodative lens refilling on capsular opacification (part 1);
2. To improve technical aspects related to the performance of cataract surgery (part 2).

Part 1 of this thesis starts with a review in **Chapter 2**, which provides an overview of current knowledge on the prevention of capsular opacification. First, biological pathways involved in CO formation are described, followed by the various approaches to interfere

with these processes. The table in this chapter provides an overview of all methods which have been tested in studies to prevent CO and methods with potential for future applications are further discussed. **Chapter 3** focuses on the effects of CO on the elasticity of the lens capsule. The stiffness of the lens is an important factor for lens accommodation. It has always been thought that CO formation is responsible for an increase in lens stiffness and a decrease in accommodative amplitude. However, the relation between the development of CO and the stiffness of the lens has never been studied previously. Additionally, this chapter demonstrates that prevention of CO also prevented stiffening of the lens. **Chapter 4** continues with the examination of different types of silicone polymers for accommodative lens refilling and the effect of silicone wettability on lens stiffness and the response of the LECs.

In **Chapter 5**, an agent which is currently used in cataract surgery was investigated for its influence in CO formation. Hyaluronan (sodium hyaluronate) is a component of the viscoelastic solutions that are used during cataract surgery to maintain the anterior chamber and facilitate IOL implantation. Our data shows the influence of hyaluronan on the development of CO. Hyaluronan is also a component in the nanogels which we used to explore methods for CO prevention. In **Chapter 6** we examine the effect of these nanogels on LECs by evaluating the effect of 14 different combinations of nanogels with attached peptides on CO formation in refilled porcine lens capsules. **Chapter 7** continues with experiments elaborating on the optimization of peptide ratios in these different nanogels in order to work towards the prevention of CO.

The second part of this thesis focuses on other aspects of cataract surgery and the required skills for performing this surgery. Since the experiments for the prevention of CO required the use of new surgical techniques, we looked into methods for proper preservation of porcine eyes in **Chapter 8**. For the development of surgical skills of young ophthalmologists it is very important to obtain a good view of the surgical field of the eye. **Chapter 9** analyzes the importance of stereoscopic vision on surgical performance with the operating microscope. Since 2001, residents in the Netherlands have to achieve a minimum score on a standardized random-dot stereoscopy test in order to be eligible for a residency in the specialty of ophthalmology.[8] However, there was little to no evidence that eye-surgical performance would suffer from a lack of stereovision. Therefore, we aimed to identify the relevance of screening for stereovision for admission to ophthalmology residency programs.

Chapter 10 discusses the results of this thesis and provides handles and ideas for future research.

References

Ament C.S., Henderson B.A., 2011. Optimizing resident education in cataract surgery. Curr. Opin. Ophthalmol. 22, 64-67.

Apple D.J., Solomon K.D., Tetz M.R., Assia E.I., Holland E.Y., Legler U.F., Tsai J.C., Castaneda V.E., Hoggatt J.P., Kostick A.M., 1992. Posterior capsule opacification. Surv. Ophthalmol. 37, 73-116.

Aslam T.M., Devlin H., Dhillon B., 2003. Use of Nd:YAG laser capsulotomy. Surv. Ophthalmol. 48, 594-612.

Awasthi N., Guo S., Wagner B.J., 2009. Posterior capsular opacification: a problem reduced but not yet eradicated. Arch. Ophthalmol. 127, 555-562.

Binenbaum G., Volpe N.J., 2006. Ophthalmology resident surgical competency: a national survey. Ophthalmology 113, 1237-1244.

Birinci H., Kuruoglu S., Oge I., Oge F., Acar E., 1999. Effect of intraocular lens and anterior capsule opening type on posterior capsule opacification. J. Cataract Refract. Surg. 25, 1140-1146.

Cui H., Webber M.J., Stupp S.I., 2010. Self-assembly of peptide amphiphiles: from molecules to nanostructures to biomaterials. Biopolymers 94, 1-18.

de Iongh R.U., Wederell E., Lovicu F.J., McAvoy J.W., 2005. Transforming growth factor-beta-induced epithelial-mesenchymal transition in the lens: a model for cataract formation. Cells Tissues Organs 179, 43-55.

Dewey S., Beiko G., Braga-Mele R., Nixon D.R., Raviv T., Rosenthal K., ASCRS Cataract Clinical Committee, Instrumentation and IOLs Subcommittee, 2014. Microincisions in cataract surgery. J. Cataract Refract. Surg. 40, 1549-1557.

Eldred J.A., Dawes L.J., Wormstone I.M., 2011. The lens as a model for fibrotic disease. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 366, 1301-1319.

Ellis-Behnke R., Jonas J.B., 2011. Redefining tissue engineering for nanomedicine in ophthalmology. Acta Ophthalmol. 89, e108-114.

Etheridge M.L., Campbell S.A., Erdman A.G., Haynes C.L., Wolf S.M., McCullough J., 2013. The big picture on nanomedicine: the state of investigational and approved nanomedicine products. Nanomedicine 9, 1-14.

Euliss L.E., DuPont J.A., Gratton S., DeSimone J., 2006. Imparting size, shape, and composition control of materials for nanomedicine. Chem. Soc. Rev. 35, 1095-1104.

Findl O., Buehl W., Bauer P., Sycha T., 2010. Interventions for preventing posterior capsule opacification. Cochrane Database Syst. Rev. 2, CD003738.

Fisher R.F., 1988. The mechanics of accommodation in relation to presbyopia. Eye (Lond) 2, 646-649.

Ge J., Wand M., Chiang R., Paranhos A., Shields M.B., 2000. Long-term effect of Nd:YAG laser posterior capsulotomy on intraocular pressure. Arch. Ophthalmol. 118, 1334-1337.

Gentleman D.J., Chan W.C., 2009. A systematic nomenclature for codifying engineered nanostructures. Small 5, 426-431.

Georgopoulos M., Menapace R., Findl O., Petternel V., Kiss B., Rainer G., 2003. After-cataract in adults with primary posterior capsulorhexis: comparison of hydrogel and silicone intraocular lenses with round edges after 2 years. J. Cataract Refract. Surg. 29, 955-960.

Glasser A., 2008. Restoration of accommodation: surgical options for correction of presbyopia. Clin. Exp. Optom. 91, 279-295.

Goldberg M., Langer R., Jia X., 2007. Nanostructured materials for applications in drug delivery and tissue engineering. J. Biomater. Sci. Polym. Ed. 18, 241-268.

Guha R., Chowdhury S., Palui H., Mishra A., Basak S., Mandal T.K., Hazra S., Konar A., 2013. Doxorubicin-loaded MePEG-PCL nanoparticles for prevention of posterior capsular opacification. Nanomedicine (Lond) 8, 1415-1428.

Haefliger E., Parel J.M., Fantes F., Norton E.W., Anderson D.R., Forster R.K., Hernandez E., Feuer W.J., 1987. Accommodation of an endocapsular silicone lens (Phaco-Ersatz) in the nonhuman primate. Ophthalmology 94, 471-477.

Hashimoto C., Kurosaka D., Uetsuki Y., 2001. Teaching continuous curvilinear capsulorhexis using a postmortem pig eye with simulated cataract. J. Cataract Refract. Surg. 27, 814-816.

Hazra S., Palui H., Vemuganti G.K., 2012. Comparison of design of intraocular lens versus the material for PCO prevention. Int. J. Ophthalmol. 5, 59-63.

Henderson B.A., Grimes K.J., Fintelmann R.E., Oetting T.A., 2009. Stepwise approach to establishing an ophthalmology wet laboratory. J. Cataract Refract. Surg. 35, 1121-1128.

Hoerenz P., 1980. The operating microscope. I. Optical principles, illumination systems, and support systems. J. Microsurg. 1, 364-369.

Huang X., Wang Y., Cai J.P., Ma X.Y., Li Y., Cheng J.W., Wei R.L., 2013. Sustained release of 5-fluorouracil from chitosan nanoparticles surface modified intra ocular lens to prevent posterior capsule opacification: an in vitro and in vivo study. J. Ocul. Pharmacol. Ther. 29, 208-215.

Ionescu A.M., Alaminos M., de la Cruz Cardona J., de Dios Garcia-Lopez Duran J., Gonzalez-Andrades M., Ghinea R., Campos A., Hita E., del Mar Perez M., 2011. Investigating a novel nanostructured fibrin-agarose biomaterial for human cornea tissue engineering: rheological properties. J. Mech. Behav. Biomed. Mater. 4, 1963-1973.

Kaiser J.M., Imai H., Haakenson J.K., Brucklacher R.M., Fox T.E., Shanmugavelandy S.S., Unrath K.A., Pedersen M.M., Dai P., Freeman W.M., Bronson S.K., Gardner T.W., Kester M., 2013. Nanoliposomal minocycline for ocular drug delivery. Nanomedicine 9, 130-140.

Klein R., Klein B.E., 2013. The prevalence of age-related eye diseases and visual impairment in aging: current estimates. Invest. Ophthalmol. Vis. Sci. 54, ORSF5-ORSF13.

Koopmans S.A., Terwee T., Barkhof J., Haitjema H.J., Kooijman A.C., 2003. Polymer refilling of presbyopic human lenses in vitro restores the ability to undergo accommodative changes. Invest. Ophthalmol. Vis. Sci. 44, 250-257.

Koopmans S.A., Terwee T., Glasser A., Wendt M., Vilupuru A.S., van Kooten T.G., Norrby S., Haitjema H.J., Kooijman A.C., 2006. Accommodative lens refilling in rhesus monkeys. Invest. Ophthalmol. Vis. Sci. 47, 2976-2984.

Koopmans S.A., Terwee T., Haitjema H.J., Deuring H., Aarle S., Kooijman A.C., 2004. Relation between injected volume and optical parameters in refilled isolated porcine lenses. Ophthalmic Physiol. Opt. 24, 572-579.

Koopmans S.A., Terwee T., van Kooten T.G., 2011. Prevention of capsular opacification after accommodative lens refilling surgery in rabbits. Biomaterials 32, 5743-5755.

Kreyling W.G., Semmler-Behnke M., Chaudhry Q., 2010. A complementary definition of nanomaterial. Nano Today 5, 165-168.

Li X., Zhang Z., Chen H., 2013. Development and evaluation of fast forming nano-composite hydrogel for ocular delivery of diclofenac. Int. J. Pharm. 448, 96-100.

Lopez-Novoa J.M., Nieto M.A., 2009. Inflammation and EMT: an alliance towards organ fibrosis and cancer progression. EMBO Mol. Med. 1, 303-314.

Lovicu F.J., Shin E.H., McAvoy J.W., 2015. Fibrosis in the lens. Sprouty regulation of TGFbeta-signaling prevents lens EMT leading to cataract. Exp. Eye Res. Epub.

Marcantonio J.M., Syam P.P., Liu C.S., Duncan G., 2003. Epithelial transdifferentiation and cataract in the human lens. Exp. Eye Res. 77, 339-346.

Marcantonio J.M., Vrensen G.F., 1999. Cell biology of posterior capsular opacification. Eye (Lond) 13, 484-488.

McCannel C.A., Reed D.C., Goldman D.R., 2013. Ophthalmic surgery simulator training improves resident performance of capsulorhexis in the operating room. Ophthalmology 120, 2456-2461.

Menapace R., 2008. Posterior capsulorhexis combined with optic buttonholing: an alternative to standard in-the-bag implantation of sharp-edged intraocular lenses? A critical analysis of 1000 consecutive cases. Graefes Arch. Clin. Exp. Ophthalmol. 246, 787-801.

Moore D.B., Harris A., Siesky B., 2010. The world through a lens: the vision of Sir Harold Ridley. Br. J. Ophthalmol. 94, 1277-1280.

Nibourg L.M., Sharma P.K., van Kooten T.G., Koopmans S.A., 2015a. Changes in lens stiffness due to capsular opacification in accommodative lens refilling. Exp. Eye Res. 134, 148-154.

Nibourg L.M., Gelens E., Kuijer R., Hooymans J.M.M., van Kooten T.G., Koopmans S.A., 2015b. Prevention of posterior capsular opacification. Exp. Eye Res. 136, 100-115.

Nieto M.A., 2011. The ins and outs of the epithelial to mesenchymal transition in health and disease. Annu. Rev. Cell Dev. Biol. 27, 347-376.

Nishi O., Nishi K., 1998. Accommodation amplitude after lens refilling with injectable silicone by sealing the capsule with a plug in primates. Arch. Ophthalmol. 116, 1358-1361.

Nishi O., Nishi K., Akaishi T., Shirasawa E., 1997. Detection of cell adhesion molecules in lens epithelial cells of human cataracts. Invest. Ophthalmol. Vis. Sci. 38, 579-585.

Nishi O., Nishi K., Wickstrom K., 2000. Preventing lens epithelial cell migration using intraocular lenses with sharp rectangular edges. J. Cataract Refract. Surg. 26, 1543-1549.

Nishi Y., Mireskandari K., Khaw P., Findl O., 2009. Lens refilling to restore accommodation. J. Cataract Refract. Surg. 35, 374-382.

Ong H.S., Evans J.R., Allan B.D., 2014. Accommodative intraocular lens versus standard monofocal intraocular lens implantation in cataract surgery. Cochrane Database Syst. Rev. 5, CD009667.

Pande M., Spalton D.J., Marshall J., 1996a. Continuous curvilinear capsulorhexis and intraocular lens biocompatibility. J. Cataract Refract. Surg. 22, 89-97.

Pande M.V., Spalton D.J., Kerr-Muir M.G., Marshall J., 1996b. Postoperative inflammatory response to phacoemulsification and extracapsular cataract surgery: aqueous flare and cells. J. Cataract Refract. Surg. 22, 770-774.

Pritchard C.D., Arner K.M., Langer R.S., Ghosh F.K., 2010. Retinal transplantation using surface modified poly(glycerol-co-sebacic acid) membranes. Biomaterials 31, 7978-7984.

Spalton D.J., 2009. Diamond jubilee of the first intraocular lens implantation? J. Cataract Refract. Surg. 35, 2040-2041.

Spalton D.J., Russell S.L., Evans-Gowing R., Eldred J.A., Wormstone I.M., 2014. Effect of total lens epithelial cell destruction on intraocular lens fixation in the human capsular bag. J. Cataract Refract. Surg. 40, 306-312.

Steinert R.F., Puliafito C.A., Kumar S.R., Dudak S.D., Patel S., 1991. Cystoid macular edema, retinal detachment, and glaucoma after Nd:YAG laser posterior capsulotomy. Am. J. Ophthalmol. 112, 373-380.

Stifter E., Menapace R., Luksch A., Neumayer T., Vock L., Sacu S., 2007. Objective assessment of intraocular flare after cataract surgery with combined primary posterior capsulorhexis and posterior optic buttonholing in adults. Br. J. Ophthalmol. 91, 1481-1484.

Strenk S.A., Semmlow J.L., Strenk L.M., Munoz P., Gronlund-Jacob J., DeMarco J.K., 1999. Age-related changes in human ciliary muscle and lens: a magnetic resonance imaging study. Invest. Ophthalmol. Vis. Sci. 40, 1162-1169.

van Kooten T.G., Koopmans S., Terwee T., Norrby S., Hooymans J.M., Busscher H.J., 2006. Development of an accommodating intra-ocular lens--in vitro prevention of re-growth of pig and rabbit lens capsule epithelial cells. Biomaterials 27, 5554-5560.

van Vreeswijk H., Pameyer J.H., 1998. Inducing cataract in postmortem pig eyes for cataract surgery training purposes. J. Cataract Refract. Surg. 24, 17-18.

Vasavada A.R., Raj S.M., Johar K., Nanavaty M.A., 2006. Effect of hydrodissection alone and hydrodissection combined with rotation on lens epithelial cells: surgical approach for the prevention of posterior capsule opacification. J. Cataract Refract. Surg. 32, 145-150.

Wang D., Guo D., Bi H., Wu Q., Tian Q., Du Y., 2013. Zinc oxide nanoparticles inhibit Ca-ATPase expression in human lens epithelial cells under UVB irradiation. Toxicol. in. Vitro. 27:2117-26.

Weeber H.A., van der Heijde R.G., 2007. On the relationship between lens stiffness and accommodative amplitude. Exp. Eye Res. 85, 602-607.

Wormstone I.M., Wang L., Liu C.S., 2009. Posterior capsule opacification. Exp. Eye Res. 88, 257-269.

Zarbin M.A., Montemagno C., Leary J.F., Ritch R., 2010. Nanomedicine in ophthalmology: the new frontier. Am. J. Ophthalmol. 150, 144-162.e2.

Zhang W., Li X., Ye T., Chen F., Sun X., Kong J., Yang X., Pan W., Li S., 2013. Design, characterization, and in vitro cellular inhibition and uptake of optimized genistein-loaded NLC for the prevention of posterior capsular opacification using response surface methodology. Int. J. Pharm. 454, 354-366.

Zhang X.H., Ji J., Zhang H., Tang X., Sun H.M., Yuan J.Q., 2000. Detection of integrins in cataract lens epithelial cells. J. Cataract Refract. Surg. 26, 287-291.

Other cited material

A. World Health Organization, Geneva, Switzerland. Available at: http://www.who.int/blindness/causes/priority/en/index1.html. Accessed July 1, 2015

B. Stilma J.S., Tan H.S., Bijlsma W.R., 2008. Modernisering Opleiding Oogheelkunde Nederland. Available at: http://www.oogheelkunde.org/professionals/opleiding/concilium2/moon-versie-mei-2008. Accessed July 8, 2015

Chapter 2

Prevention of posterior capsular opacification

Lisanne M. Nibourg, Edith Gelens, Roel Kuijer, Johanna M.M. Hooymans, Theo G. van Kooten, and Steven A. Koopmans

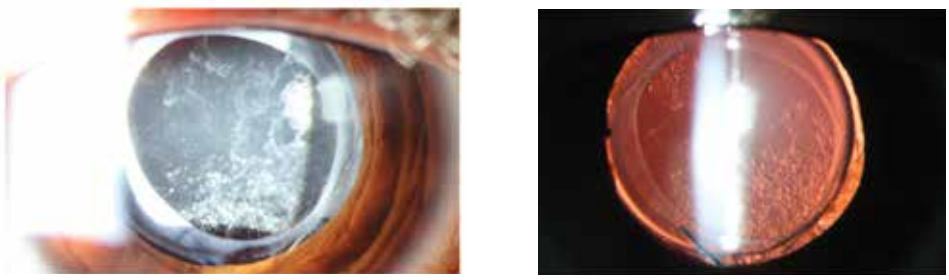
This chapter is an edited version of the manuscript: Nibourg L.M., Gelens E., Kuijer R., Hooymans J.M.M., van Kooten T.G., Koopmans S.A., 2015. Prevention of posterior capsular opacification. Experimental Eye Research. 136, 100-115. (Re-use permitted)

Abstract

Posterior capsular opacification (PCO) is a common complication of cataract surgery. The development of PCO is due to a combination of the processes of proliferation, migration, and transdifferentiation of residual lens epithelial cells (LECs) on the lens capsule. In the past decades, various forms of PCO prevention have been examined, including adjustments of techniques and intraocular lens materials, pharmacological treatments, and prevention by interfering with biological processes in LECs. The only method so far that seems effective is the implantation of an intraocular lens with sharp edged optics to mechanically prevent PCO formation. In this review, current knowledge of the prevention of PCO will be described. We illustrate the biological pathways underlying PCO formation and the various approaches to interfere with the biological processes to prevent PCO. In this type of prevention, the use of nanotechnological advances can play a role.

2.1 Introduction

Posterior capsular opacification (PCO) is a common complication of cataract surgery. In cataract surgery, the crystalline lens fibers from the natural opaque lens nucleus and cortex are removed, followed by implantation of an intraocular lens (IOL) in the remaining lens capsular bag. Within two to five years after this surgery, PCO can cause a decreased visual acuity in 20 to 40 percent of patients (Figure 1) (Awasthi et al., 2009).



A B

FIGURE 1. *Slit lamp photograph under direct illumination (A) and retro illumination (B) of an eye showing posterior capsular opacification six years after cataract surgery.*

PCO is a fibrotic condition initiated by an inflammatory response due to tissue trauma caused by cataract surgery and combined with a foreign body reaction towards the implanted IOL. The eye lens has been considered to be a model for organ fibrosis in general because of the PCO response (Eldred et al., 2011). The PCO response is a consequence of proliferation, migration, and transdifferentiation of lens epithelial cells (LECs) that are normally situated in a single layer on the inner side of the anterior lens capsule and remain in the capsular bag after cataract surgery (Apple et al., 1992, Marcantonio and Vrensen, 1999, Raj et al., 2009). Surgeons remove most of these LECs during cataract surgery, however, there are always residual cells in the capsular bag. Proliferation rates of these residual cells are highest three to four days after surgery and they are age-dependent, since younger patients are more at risk for PCO (Dawes et al., 2013, Maltzman et al., 1989). The exact mechanism which initiates the proliferation is unknown. It could be related to the change in extracellular matrix (ECM) components and growth factors due to the inflammatory response (Eldred et al., 2011, Nishi, 2012, Oharazawa et al., 1999, Saika et al., 2002). The second process is the migration of LECs to the posterior capsule. Migration over the posterior lens capsule towards the posterior visual axis is possible due to cell adhesion molecules involved in formation and disruption of cell-cell and cell-ECM contacts, such as intracellular adhesion molecule-1 (ICAM-1), various integrin-ligands, and CD44 (McLean et al. 2005, Nishi et al. 1997, Saika et al. 1998, Zhang et al. 2000). Furthermore, during the formation of PCO, LECs transdifferentiate. LECs in the equator can differentiate into cells which resemble normal crystalline lens fibers (Marcantonio and Vrensen, 1999, Wormstone et al., 2009). LECs on the anterior side of the capsule can undergo transdifferentiation to myofibroblasts resulting in PCO (Apple et al., 1992, de Iongh et al., 2005).

Epithelial to mesenchymal transformation (EMT) is the process of transdifferentiation of epithelial cells into mesenchymal cells (Nieto, 2011). Mesenchymal cells have migratory and invasive properties. As a consequence of the transdifferentiation of LECs to myofibroblasts, which contain α-smooth muscle actin (αSMA) and have contractile properties, the lens capsule surface can become wrinkled (Marcantonio et al., 2003, Wormstone, 2002). When these wrinkles are situated in the visual axis they cause visual disturbances. The specific mechanisms of EMT in PCO formation are described in detail in section 2.2.

PCO in the central visual axis is usually treated with a neodymium: YAG (Nd:YAG) laser capsulotomy of the posterior lens capsule and occasionally with a surgical

posterior capsulotomy. These treatments result in an opening in the central posterior lens capsule, providing a clear visual axis. Surgical posterior capsulotomy concerns an invasive procedure with additional risks for the patient. Similarly, complications have been described for the Nd:YAG laser capsulotomy, including damage to the IOL, elevation of intraocular pressure, cystoid macular edema, and an increased incidence of retinal detachment (Aslam et al., 2003, Awasthi et al., 2009, Ge et al., 2000, Steinert et al., 1991, Trinavarat et al., 2001). Beside the complications, the considerable high procurement costs of a Nd:YAG laser make laser capsulotomy not universally available for all patients suffering from PCO. Hence, there is a strong need to prevent PCO instead of treating it with a laser capsulotomy.

In this review current knowledge on mechanisms of EMT is summarized and possible ways to interfere in these processes for the prevention of PCO are indicated. In the last section we will broach the potential of new strategies using nanotechnological advances for the prevention of PCO.

2.2 Mechanisms of epithelial to mesenchymal transformation

Epithelial to mesenchymal transformation is the process of transdifferentiation of epithelial cells into mesenchymal cells. This process can be triggered by an inflammatory response (Lopez-Novoa and Nieto, 2009), such as the response in the eye after cataract surgery (Wormstone et al., 2009). Damaged ocular tissue releases chemokines which attract cells of the immune system in order to remove damaged tissue and to facilitate subsequent tissue repair. Clinically, this is characterized by the presence of cells and flare in the anterior chamber (Pande et al., 1996b). During the wound healing reaction many cytokines (e.g. interleukin-1β and tumor necrosis factor (TNF)-α) and prostaglandins play key roles in modulation of the tissue response (de Iongh et al., 2005, Marcantonio and Vrensen, 1999). The wound healing reaction also influences the proliferation, migration, and differentiation of the LECs resulting in PCO. The exact mechanisms relating the development of PCO with postsurgical inflammation are not yet fully understood, however, the relation between inflammation and EMT has been described in other tissues (Kalluri and Neilson, 2003, Lopez-Novoa and Nieto, 2009, Wu et al., 2009). EMT is active in various processes like morphogenesis, organogenisis (by stem cells), homeostasis (in tissue regeneration, inflammation, and wound healing), and in diseases (like organ fibrosis

and cancer) (Marcantonio and Vrensen, 1999, Marcantonio et al., 2003, Nieto, 2011). The process of EMT is activated during the progression of cancer and organ fibrosis, and this process is an important step in the metastatic cascade of epithelial tumors.

After discoveries concerning the general process of EMT, biological pathways specifically involved in the formation of PCO were identified that enable new approaches for PCO prevention. Since many growth factors and other signaling molecules are involved in the development of PCO, they present potential targets for therapy.

The role of TGF-β

The growth factor family of TGF-β has an important role in PCO development, as TGF-β signaling has been implicated in the transdifferentiation of LECs and fibrosis of the lens capsule (de Iongh et al., 2005, Eldred et al., 2011, Marcantonio and Vrensen, 1999, Zheng et al., 2012). The anterior side of the human capsular bag is normally covered by hexagonally shaped LECs. TGF-β signaling in the capsular bag can induce expression of ECM proteins, such as laminin, fibronectin, vitronectin, proteoglycans, collagen I, collagen III, and tenascin (Ishibashi et al., 1994, Linnola et al., 2000a, Lovicu et al., 2002, Olivero and Furcht, 1993, Saika et al., 2003, Tanaka et al., 2010); and intermediate filaments or microfilaments, like desmin, α-smooth muscle actin (αSMA), and tropomyosin (Kubo et al., 2013, Lovicu et al., 2002, Marcantonio et al., 2003). TGF-β has different isoforms, of which TGF-β2 has the highest expression in lens tissue and aqueous humor. Apart from TGF-β2 lens tissue can also express TGF-β1 and TGF-β3 (Saika et al., 2000, Wallentin et al., 1998).

The latent form of TGF-β is normally present in the aqueous humor (Eldred et al., 2011). TGF-β activation is induced in reaction to trauma . Signaling by TGF-β starts with the binding of TGF-β to type I and type II receptor serine/threonine kinases on the cell surface (Figure 2). The type I and II receptors are brought together and receptor II phosphorylates the receptor I kinase domain, resulting in a signal into the cell by phosphorylation of Smad proteins (de Iongh et al., 2005, Shi and Massague, 2003). These intracellular Smad proteins can be divided into three functional groups: receptor-activated Smads (R-Smads), common-mediator Smad (co-Smad), and inhibitory or antagonistic Smads (I-Smads). Many studies evaluated the specific Smad signaling pathways of TGF-β in LECs (Li et al., 2014, Li et al., 2011, Saika et al., 2004, Wang et al., 2013b, Wormstone et al., 2004). The R-Smads Smad2 and Smad3 are expressed in response to TGF-β and they regulate the transcription of TGF-β target genes (Li et al., 2011, Saika et al., 2004). When Smad2 and

Smad3 are phosphorylated they interact with the co-Smad Smad4 and a Smad complex will be formed (Wang et al., 2013b). This complex is transported into the nucleus of the LEC and will activate transcription of TGF-β target genes (Dawes et al., 2007, de Iongh et al., 2005).

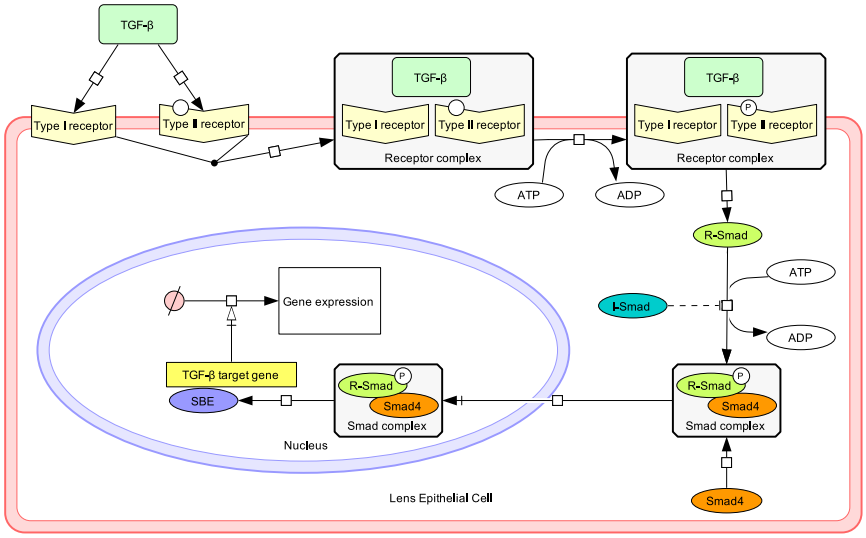


FIGURE 2. *Schematic overview of TGF-β/Smad signaling in LECs. Following trauma, TGF-β is activated and a TGF-β receptor complex will be formed. A signal is send into the cell by phosphorylation of a R-Smad and the R-Smad can interact with the co-Smad (Smad4) to form a Smad complex. This complex will be transported into the nucleus to activate transcription of TGF-β target genes. The formation of a Smad complex can be inhibited by I-Smad proteins. Figure made with CellDesigner (Funahashi et al., 2003).*

In addition to the Smad dependent signaling pathway, recent evidence suggests that also other Smad independent pathways are involved in the transmission of TGF-β signals in LECs (Figure 3). TGF-β has been shown to activate Rho GTPases, phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK); also extracellular signal-regulated kinase (ERK) and Notch pathways were recently related to TGF-β signaling (Chen et al., 2014, Cho and Yoo, 2007, Cho et al., 2007, de Iongh et al., 2005, Kardassis et al., 2009, Lovicu and McAvoy, 2001, Lovicu et al., 2011). The TGF-β receptor complex directly activates Rho GTPases by phosphorylation (Kardassis et al., 2009). Rho GTPases are responsible for regulating the actin cytoskeleton structure, and activation of this pathway in LECs has been related to the formation of stress fibers and myofibroblasts

in PCO (Cho and Yoo, 2007). The transcriptional repressor snail activates the PI3K/Akt signaling pathway and was found to be an early-response gene of TGF-β signaling (Cho et al., 2007). Snail expression is associated with suppression of the epithelial phenotype in tumor cells and has been identified as a marker for EMT in LECs. In response to TGF-β release, matrix metalloproteinase (MMP)-2 and MMP-9 are expressed and are involved in EMT-related matrix contraction and remodeling through the MAPK pathway (Sivak and Fini, 2002). Additionally, in LECs from human cataract samples the MAPK pathway was found to be responsible for the expression of the EMT marker slug, a member of the snail superfamily (Choi et al., 2007). The intracellular signaling molecules ERK1/2 are involved in cell cycle regulation and differentiation, and were recently found to interact with TGF-β/Smad signaling in LECs (Chen et al., 2014). ERK1/2 signaling was shown to be activated in TGF-β2 induced EMT, and blockage of ERK1/2 in human LECs resulted in an absence of TGF-β2 induced EMT markers (Chen et al., 2014), whereas blockage of Smad2/3 did not result in inhibition of ERK1/2 signaling by TGF-β2.

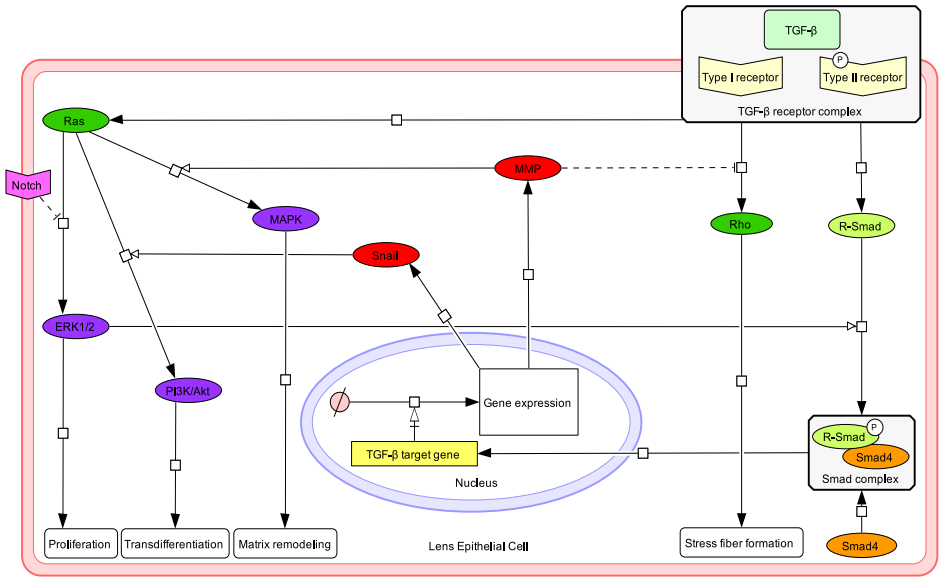


FIGURE 3. *Schematic overview of other signaling pathways involved in TGF-β signaling in LECs. Interactions between the TGF-β/Smad pathway and other Smad independent pathways are shown. Other pathways include Rho, PI3K/Akt, MAPK, ERK1/2, and Notch. Figure made with CellDesigner (Funahashi et al., 2003).*

The association of the Notch pathway with TGF-β signaling in EMT was also shown. Notch proteins are transmembrane receptors mainly involved in cell-cell signaling. Blockage of the Notch pathway in LECs resulted in TGF-β2-induced activation of ERK1/2 signaling (Chen et al., 2014). Thus the Notch pathway seems to protect normal epithelial behavior of LECs.

The wide influence of TGF-β in LEC signaling and the variety of involved signaling pathways make TGF-β a target of great potential for the prevention of PCO.

Other growth factors

Apart from TGF-β, other signaling molecules have been described to be involved in PCO development, such as fibroblast growth factor (FGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), bone morphogenetic protein (BMP), Wingless-related integration site (Wnt), and tumor necrosis factor (TNF)-α (Choi et al., 2004, Huang et al., 2011, Iyengar et al., 2009, Lee et al., 2007, Mansfield et al., 2004, Martinez et al., 2009, Meacock et al., 2000b, Prada et al., 2000, Tanaka et al., 2004, Wertheimer et al., 2013, Zhang et al., 2001). Figure 4 shows a schematic overview of the interaction between TGF-β signaling and other pathways associated with PCO development.

The growth factor FGF has a role in PCO by inducing LEC proliferation and the production of the EMT markers αSMA, collagen type I, and fibronectin (Lovicu and McAvoy, 2001, Mansfield et al., 2004). It was found that TGF-β2 alone has a suppressive effect on the LEC proliferation (Saika et al., 2001). However, with the addition of FGF-2 this suppressive effect is overtaken by the stimulating effect of FGF-2, resulting in LEC proliferation (Tanaka et al., 2004). This shows the complexity of the signaling systems and raises the question if targeting TGF-β alone (as in subsection 2.3 page 46) will result in complete prevention of PCO formation. Choi et al. (2004) identified the role of the growth factor HGF in LEC proliferation. HGF was shown to induce LEC proliferation *in vitro*. This proliferation was induced by cyclin D1 protein, which is regulated by the MAPK and PI3K/AKT pathways. The mature form of HGF is a glycoprotein that can be bound by heparin. The glycosaminoglycan heparin was found to inhibit LEC proliferation probably because of its affinity to bind growth factors (Xie et al., 2003). The influence of EGF was stated by Huang et al. (2011) by the use of EGFR targeted siRNA in human LECs *in vitro* resulting in inhibited proliferation. Iyengar et al. (2009) performed a comparative study of FGF-2, EGF, IGF-I, and PDGF-A in which they found evidence for the effect of these growth

factors on inducing proliferation in LECs. They observed that it is always a combination of growth factors which is responsible for induction of proliferation and that FGF is a key regulator. In another study, the growth factor PDGF was found to induce migration of LECs via the PI3K/Akt signaling pathway (Xiong et al., 2010). CTGF was found to be expressed as a TGF-β2 response gene (Wormstone et al., 2004) and was recently found to be involved in EMT-related ECM remodeling in human LECs via the SMAD signaling pathway (Ma et al., 2014). CTGF induces activation of the R-Smads, whereas silencing of CTGF resulted in inhibition of TGF-β2-induced phosphorylation of the R-Smads. Next to CTGF, Ma et al. (2014) reported a similar effect of Gremlin, an endogenous BMP-antagonist, on TGF-β2 signaling in human LECs *in vitro*. Gremlin interacts with BMP-receptors resulting in inhibition of BMP signaling. Since BMPs were found to antagonize TGF-β signaling, blockage of BMP signaling can result in EMT (Lee et al., 2007, Ma et al., 2014, Saika et al., 2006). Wnt is a factor that regulates epithelial transdifferentiation by the Wnt/β-catenin pathway (Martinez et al., 2009). TGF-β was found to stimulate Wnt expression during cataract formation (Chong et al., 2009), and the expression of Wnt has recently been related to the stimulation of EMT-induced migration and proliferation in human LECs *in vitro* (Bao et al., 2012). Finally, TNF-α gene expression was found in LECs of capsule samples post cataract surgery (Prada et al., 2000). Zhang et al. (2001) described that TNF-α induces the transcription and translation of type IV collagen, which is present in the normal lens but may also be involved with EMT-related ECM deposition. However, the role of TNF-α in the development of PCO is still obscure.

Integrins

Another target in PCO prevention is integrin signaling. Integrins are receptors mainly involved in cell-ECM interactions and many integrin members are present in LECs (McLean et al., 2005, Worthington et al., 2011, Zhang et al., 2000). A variety of signaling pathways (including Wnt, Rho, PI3K/Akt, and MAPK) and growth factor receptors (like IGF and FGF receptors) can be affected by cell adhesion via integrins (Wederell and de Iongh, 2006). In PCO development, changes in integrin-ECM interactions are associated with transdifferentiation of LECs (Mamuya et al., 2014, Walker and Menko, 2009), and integrins were found to be activators for TGF-β, present in the latent form in the ECM (Dawes et al., 2007, Mamuya et al., 2014, Worthington et al., 2011). Integrins can bind a large variety of ligands, including ECM components and other molecules involved in cellular signaling (Wederell and de Iongh, 2006).

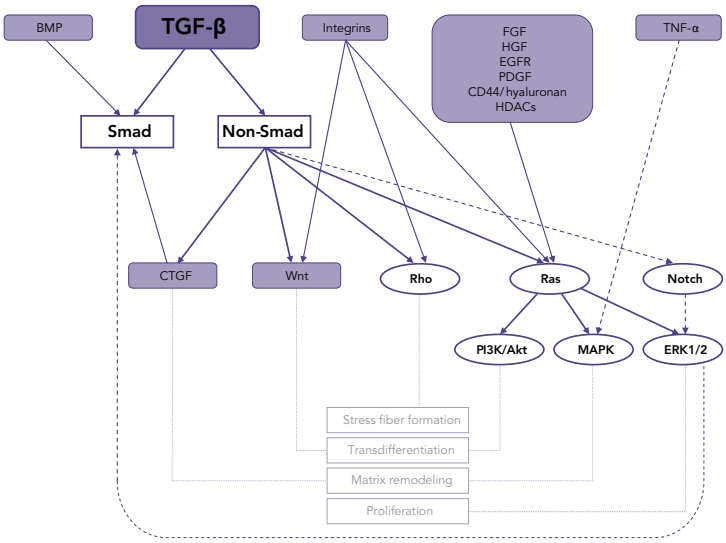


FIGURE 4. *Schematic overview of interactions between TGF-β signaling and other pathways associated with PCO development. The graph shows possible interactions and their related LEC response.*

Integrin-linked kinase (ILK) can regulate integrin signaling by binding to the β-subunit of the integrin and to a growth factor receptor (Wederell and de Iongh, 2006). ILK regulates cellular processes via PI3K/Akt, MAPK, and Wnt pathways. Deletion of ILK in mice resulted in capsular rupture, abnormal lens formation, disorganization of LECs, reduced LEC proliferation, and LEC death (Cammas et al., 2012, Teo et al., 2014). Therefore, ILK could possibly be a target in influencing LEC signaling in PCO.

CD44/hyaluronan

The CD44/hyaluronan pathway has been associated with TGF-β signaling (Acharya et al., 2008). CD44 is the receptor for hyaluronan but can also interact with growth factors and MMPs. Both CD44 and hyaluronan were found in human anterior capsules obtained during cataract surgery (Saika et al., 1998). Canine LECs treated with different concentrations of hyaluronan showed a dose-dependent increase in CD44 expression and increased LEC migration (Chandler et al., 2012). This indicates the possible role for CD44 and hyaluronan in the migration of LECs. Interestingly, viscoelastics which are used during cataract surgery all contain hyaluronan. Normally, during cataract surgery the viscoelastics are removed after IOL implantation. However, it is likely that there is contact between

LECs and viscoelastics during the surgery, and the probability that some viscoelastics will be left behind in the lens capsule is high. So this could also be a factor to take into account for future approaches in PCO prevention.

Histone deacetylases

Histone deacetylase (HDAC)-mediated epigenetic mechanisms play a central role in cell cycle regulation and were recently found to be involved in EMT in LECs (Chen et al., 2013). Little is known yet about the processes underlying epigenetic mechanisms. Up to now, 18 HDAC isoforms have been described, which can be divided into four different classes. Isoforms from class I and II HDACs were found to be upregulated in TGF-β2-induced EMT in LECs. Inhibition of HDACs resulted in suppressed phosphorylation of Smad2 and inhibited LEC proliferation by suppression of P13K/Akt, MAPK, and ERK1/2 pathways (Chen et al., 2013), indicating a role for HDAC inhibitors in PCO prevention.

2.3 Prevention of posterior capsular opacification

Targets for PCO prevention were discussed in section 2.2. In this part we focus on general measures, pharmacological measures, and non-pharmacological measures which were evaluated in various experimental models.

General measures

Many measures are known to reduce the formation of PCO. The material and the design of the implanted IOL influence the extent of PCO which develops postoperatively (Hazra et al., 2012b, Hollick et al., 2000, Leishman et al., 2012, Ollerton et al., 2012). For example, acrylate IOLs bind components of extracellular matrix proteins to the IOL surface, promoting capsular adhesion. These proteins bind to the LECs and the lens capsule, resulting in a firm adhesion between IOL and lens capsule which reduces migration of LECs between IOL and lens capsule (Linnola et al., 2000a, Linnola et al., 2000b). Another example is the sharp edge of the optic of several IOLs. Lenses with a sharp optic edge result in less PCO compared with round edged IOLs. It has been demonstrated that the sharp edge provides a mechanical barrier for migration of the LECs to the posterior lens capsule (Findl et al., 2010, Hazra et al., 2012b, Nishi et al., 2000). Surgical techniques can also influence the formation of PCO (Apple et al., 1992, Awasthi et al., 2009, Wormstone

et al., 2009). From all methods used to open the lens capsule, a curvilinear continuous capsulorhexis (CCC) to open the anterior capsule results in the lowest amount of PCO (Birinci et al., 1999, Pande et al., 1996a). By using cortical cleaving hydrodissection during surgery, the lens capsule is separated from the lens fibers (Vasavada et al., 2006a). When this step is combined with rotation of the IOL after implantation, it is possible to remove more residual cortical lens fibers together with some LECs from the capsule than without these steps (Vasavada et al., 2006b). Residual cortical lens fibers can also be removed by extensive bimanual irrigation and aspiration (Peng et al., 2000). Other studies have examined the effect of polishing or scraping the anterior capsule on the formation of PCO, but although more LECs were removed, it did not prevent the proliferation of residual LECs (Liu et al., 2010, Shah et al., 2009), indicating that any residual LECs are perfectly able to host a full PCO response.

Although the previously described techniques may slow down the development of PCO, they do not prevent it completely. However, when the posterior lens capsule is removed during surgery by a posterior CCC, migration of LECs into the visual axis may be prevented for a lifelong period (Stifter et al., 2007). A posterior CCC may also be combined with the bag-in-the-lens principle, in which the anterior and posterior capsulorhexis are anchored around the specially designed IOL (Tassignon and Dhubhghaill, 2014). However, the technique is not widely used yet, as performing a posterior capsulorhexis may increase the risk of vitreous loss (Georgopoulos et al., 2003, Menapace, 2008).

Pharmacological measures

The prevention of PCO by pharmacological agents has been studied extensively (Awasthi et al., 2009, Walker, 2008). Chemical agents can interfere with different processes and so have distinctive mechanisms of action. The list in Table 1 provides an overview of the various agents and methods which have been used for PCO prevention. Due to the high safety standards for using pharmacological agents in the eye, the focus is nowadays on non-toxic agents for treating PCO that can interfere with biological processes while maintaining the integrity of the lens capsule and other ocular tissues.

Experimental models

The majority of the described agents were tested in animal models, *ex vivo* tissue culture, or *in vitro* cell culture systems (Table 1). Proliferation rates and transdifferentiation of LECs can be microscopically monitored in cell cultures. In cell cultures, there are two options for

obtaining cells: use of primary cells directly harvested from human lenses or continuous cell lines. Primary human LEC culture will provide a better model for PCO prevention compared to continuous cell lines, since primary LECs are directly derived from the human lens capsule and have the original epithelial phenotype at harvest. Immortalized continuous LEC lines are more stable and can provide information about cellular processes, but are further from the human situation since a variety of cells are present. Besides, continuous LEC lines show transformed cells in part of the cell population (Ibaraki et al., 1998). In *ex vivo* tissue cultures, the whole lens or (parts of) the lens capsule from animal or human origin can be cultured, which contributes to a greater cellular variety in the system. Tissue culture from human donor eyes provides a model closest to the clinical situation in humans and can give information about LEC migration, proliferation, and transdifferentiation. However, issues resulting from relatively long preservation times may cause changes in the viability of cells. In *in vivo* animal models, a whole eye model is used, providing the best cellular variety. Animal studies are an essential part of research in the preclinical phase, showing the full range of cellular responses in LEC (proliferation, migration, and transdifferentiation) in combination with exposure to the inflammatory response induced by the cataract surgery. Without extensive tests in relevant experimental animal models, methods for PCO prevention will never be allowed to enter the clinical research stage. However, there are many (known and unknown) differences between animal models and the human situation. Therefore, it is not surprising that positive results from animal studies are no guarantee for clinical success and many methods tested in animal models will not pass the preclinical phase. An example of differences between species was given in a study by Koopmans et al. (2014), where no treatment effect was found in a monkey lens model, compared to prevention of PCO in rabbit lenses using the same treatment.

Experimental techniques

Agents tested *in vivo* can be delivered to the LECs in different ways. For instance, during cataract surgery, agents may be added to the irrigating solution, dissolved in viscoelastics, or small spheres or particles may be used to obtain a sustained delivery system. Coating of a substance on the implanted IOL is also a method to deliver drugs (Davis et al., 2012, Duncan et al., 1997, Koopmans et al., 2011, Kugelberg et al., 2010, Liu et al., 2009, Nishi et al., 1995, Pei et al., 2013, Totan et al., 2008, van Kooten et al., 2006).

Target cell cycle regulation

1. Anti-proliferation	(author, experimental model)
Cytostatic drugs	
Anti-metabolites	
5-fluorouracil	(Duncan, 2007 (EVH); Fernandez, 2004 (IVA); Huang, 2013 (CL/IVA))
Methotrexate	(Koopmans, 2014 (IVA); Sternberg, 2010 (EVH/IVA))
Cytotoxic antibiotics	
Actinomycin D	(Koopmans, 2011-2014 (IVA); van Kooten, 2006 (CPA/EVA); Sternberg, 2010 (EVH/IVA))
Calcimycin	(Geissler, 2001 (CL/EVA/EVH))
Cycloheximide	(Koopmans, 2011 (IVA); van Kooten, 2006 (CPA/EVA))
Doxorubicin	(Guha, 2013 (IVA))
Mitomycin C	(Fernandez, 2004 (IVA); Inan, 2001a-2001b (IVA); Kim, 2007 (IVA))
Other cytostatics	
Colchicine	(Legler, 1993 (IVA))
Paclitaxel	(Koopmans, 2014 (IVA))
TGF-β inhibitors	
Anti-TGF-β antibody	(Sun, 2013 (CL))
CAT-152	(Wormstone, 2004 (CL))

2. Anti-inflammatory	
NSAIDs	
Celecoxib	(Chandler, 2007 (CPA/EVA); Davis, 2012 (EVA))
Diclofenac sodium	(Inan, 2001b (IVA))
Indometacin	(Nishi, 1995 (IVA))
Rofecoxib	(Chandler, 2007 (CPA/EVA))
Steriods	
Dexamethasone	(Inan, 2001b (IVA); Kugelberg, 2010 (IVA))
Immunosuppressivants	
Cyclosporin A	(Cortina, 1997 (CPH); Totan, 2008 (IVA); Pei, 2013 (IVA))
Rapamycin	(Liu, 2009-2010 (CPA/IVA))
Anticoagulants	
Heparin	**(Maedel, 2013 (IVH); Ronbeck, 2009 (IVH); Wejde, 2003 (IVH);** Xie, 2003 (IVA))

3. Apoptosis inducing	
Cytotoxins	
Immontoxins	
FGF2-saporin	(Behar-Cohen, 1995 (IVA))
MDX-RA (Ricin A)	**(Clark, 1998 (IVH); Meacock, 2000 (IVH))**
Polylysin-saporin	(Bretton, 1999 (IVA))
Sponge-derived	
Latrunculin B	(Sureshkumar, 2012 (EVH))
Gene therapy	
Transfer β-galactosidase gene	(Coudrec, 1999 (CPA/IVA); Malecaze, 1999 (CPA/IVA))
Transfer BMP-7	(Saika, 2006 (IVA))
Transfer HSVtk gene	(Coudrec, 1999 (CPA/IVA); Malecaze, 1999 (CPA/IVA); Yang, 2012 (CL))
Target snail	(Li, 2013a (CL))
Target TGF-β receptor	(Zheng, 2012 (CL))
Osmotic effective solutions	
Distilled (deionized) water	(Crowston, 2004 (EVH); Duncan, 2007 (EVH); Fernandez, 2004 (IVA); Kim, 2007 (IVA); **Rabsilber, 2007 (IVH); Rekas, 2013 (IVH))**

Target cell cycle regulation					
1. Anti-proliferation	(author, experimental model)	2. Anti-inflammatory		3. Apoptosis inducing	
Pirfenidone	(Yang, 2013 (CL))			NaCl	(Duncan, 2007 (EVH))
Zebularine	(Zhou, 2011-2012 (CL))			**Detergents**	
Rho inhibitors				Triton X-100	(Maloof, 2005 (IVA))
H-7	(Sureshkumar, 2012 (EVH))			**Irradation**	
Lovastatin	(Urakami, 2012 (CPA))			β-irradation isotope P-32	(Joussen, 2001 (CPA/IVA))
EGF inhibitors				Ultraviolet B	(Wang, 2013a (CL))
Erlotinib	(Wertheimer, 2013-2014 (CL))			**Iontophoresis**	
Histone deacetylase inhibitors				Acetic acid	(Fernandez, 2004 (IVA))
Trichostatin A	(Chen, 2013 (CL); Xie, 2014 (CPA/CPH))			**Photodynamic therapy**	
Vorinostat	(Xie, 2014 (CPA/CPH))			Bacteriochlorin A	(van Tenten, 2002 (IVA))
Metabolites				Indocyanin green	(Melendez, 2005 (CL))
Retinoic acid	(Inan, 2001a (IVA))			Tripan blue	(Melendez, 2005 (CL))
Tripan blue	**(Sharma, 2013 (IVH))**			**SERCA inhibitors**	
Tyrosine kinase inhibitor				Thapsigargin	(Duncan, 1997-2007 (EVH))
Genistein	(Zhang, 2013 (CL))				
Local anesthetics					
Lidocaine	(Vargas, 2003 (EVA))				

Anti-migration and anti-adherence

Matrix metalloproteinase inhibitors

Caffeic acid phenethyl ester (CAPE)	(Hepsen, 1997 (IVA); Koopmans, 2014 (IVA))
Ethylenediaminetetraacetic acid (EDTA)	(Fernandez, 2004 (IVA); Hazra, 2012a (IVA); Inan, 2001b (IVA); Nishi, 1996-1997 (IVA))
Ilomastat / GM 6001	(Awasthi, 2008 (CL/CPH/EVH))
Nobiletin	(Miyata, 2013 (CL))
Secreted protein acidic and rich in cysteine (SPARC)	(Gotoh, 2007 (CPA))

Proteasome inhibition

Disulfiram	(Sternberg, 2010 (EVH/IVA))
MG132	(Awasthi, 2006-2008 (CL/CPH/EVH))

Integrin anagonists/disintegrins

RGD peptide	(Inan, 2001b (IVA); Nishi, 1997 (IVA))
Salmosin	(Kim, 2002 (CPA/IVA))

Calcium antagonists

Mibefradil	(Weidmann, 2008 (CL/EVH))

Experimental models:

CL	Cell line (human)
CPA	Cell primary animal
CPH	Cell primary human
EVA	Ex vivo animal
EVH	Ex vivo human
IVA	In vivo animal
IVH	In vivo human

TABLE 1. *List of agents examined in studies for PCO prevention. The agents are first divided by their point of action in the process of PCO formation and subsequently by their working mechanism. The experimental models are indicated by using abbreviations which are explained in the box in the table. The in vivo human (IVH) model is highlighted in bold to emphasize that only a few treatments have been applied in patients.*

The potential of a drug for the use in PCO prevention is not only dependent on the primary effect on LECs. The effect of the drug on other ocular tissues is equally important. Especially toxicity to the corneal endothelium is an undesired side effect as it results in decompensation and opacification of the cornea. To diminish damage to other ocular tissues sealed-capsule irrigation may be a solution which prevents such side effects (Kim et al., 2007, Koopmans et al., 2011, Maloof et al., 2003, Rabsilber et al., 2007, Rekas et al., 2013). With this technique the capsular bag is isolated from the rest of the eye during the irrigation with pharmacological agents, allowing the agents to affect the LECs without damage to tissues outside the lens capsule (Maloof et al., 2003). Drawbacks of this technique are the possible leakage when the capsule is not properly sealed and the additional risks of manipulating a large device in the anterior chamber on e.g. corneal endothelium.

Cytostatic drugs

Many studies evaluated the effect of cytostatic drugs to prevent PCO (Table 1). The anti-metabolite 5-fluorouracil (5-FU) has been reported to reduce proliferation of LECs (Duncan et al., 2007, Fernandez et al., 2004). In a recent study, sustained-release of 5-FU by nanoparticles was also found to decrease LEC proliferation and induce apoptosis *in vivo* in rabbit lenses (Huang et al., 2013). Several studies have revealed the effectiveness of the cytotoxic antibiotic mitomycin C in the prevention of PCO (Fernandez et al., 2004, Inan et al., 2001b, Kim et al., 2007). Treatment with another cytotoxic antibiotic, actinomycin D, was found to reduce the development of PCO in rabbit lenses (Koopmans et al., 2011). Sternberg et al. (2010) performed *ex vivo* experiments in human capsulorhexes and found induced LEC apoptosis with actinomycin D or methotrexate therapy. A combination therapy of methotrexate and actinomycin D showed complete absence of PCO in rabbit experiments *in vivo* (Sternberg et al., 2010). Yet in an *in vivo* experiment in a monkey, combination therapy with actinomycin D, methotrexate, and caffeic acid phenethyl ester (see subsection 2.3 page 48) did not show PCO prevention (Koopmans et al., 2014). Furthermore, with these cytotoxic agents, toxic side effects to the corneal endothelium were described due to passage of the agents to the anterior chamber. Thus, the use of cytotoxic drugs in the eye remains controversial and problems regarding side effects need to be addressed first (see sealed-capsule irrigation, subsection 2.3 page 40-44).

Anti-inflammatory drugs

Chandler et al. (2007) found an increased expression of cyclooxygenase-2 (COX-2) in the normal canine lens and in LECs which underwent an EMT reaction. In *ex vivo* experiments, the treatment with the selective COX-2 inhibitors celecoxib and rofecoxib decreased the COX-2 expression in the canine capsules, resulting in decreased migration and proliferation, and increased apoptosis rates of LECs. Importantly, these effects were based on the pharmacological action of the COX-2 inhibitors and not on nonspecific toxicity. Yet the inhibitors were used in an *ex vivo* model and *in vivo* effects may be different. Davis et al. (2012) also studied celecoxib release from celecoxib incubated IOLs in canine lens capsules. In *ex vivo* experiments, they found significant less LEC growth in the group with treated IOLs compared with the control group. The immunosuppressant rapamycin was found to inhibit proliferation, migration, and fibronectin secretion in LECs (Liu et al., 2010). When coated on an IOL, rapamycin decreased the formation of PCO in rabbits (Liu et al., 2009). Cyclosporin A (CsA) belongs to the same family of immunosuppressive drugs as rapamycin and was found to inhibit human LEC proliferation *in vitro* (Cortina et al., 1997). Totan et al. (2008) investigated the effectiveness of CsA for the prevention of PCO in rabbits. Their conclusion was that CsA seems to be a possible inhibitor of PCO in rabbits, since the proliferation activity was significantly reduced in the capsules treated with CsA. A recent study of Pei et al. (2013) involved sustained-delivery of CsA to investigate the prevention of PCO in rabbits. CsA was measured in the anterior chamber untill 10 weeks post cataract surgery, and in the treatment group there was less PCO demonstrated by histopathological and electron microscopic observations.

Studies testing heparin-coated IOLs for PCO prevention reported controversial data (Ronbeck et al., 2009, Wejde et al., 2003). One study revealed that the use of a heparin delivery system implanted in the posterior capsule of rabbits was able to prevent PCO (Xie et al., 2003), whereas a recent randomized control study showed that the postoperative intraocular inflammatory response and PCO rates in patients with increased risk for intraocular inflammation did not differ between IOLs coated with heparin and IOLs without coating (Maedel et al., 2013). Thus, heparin-coating does not appear to be effective in PCO prevention.

Taken together, these findings suggest that NSAIDs and immunosuppressants have potential to reduce PCO formation. Especially their potential in sustained-delivery drug systems, which provide a lower drug concentration for a longer period of time,

are promising. Moreover, the combination of anti-inflammatory properties with PCO prevention in one agent is to be preferred.

Interfering with TGF-β signaling

Recently, Sun et al. (2013) tested a polyclonal anti-TGF-β antibody coating on IOLs in LECs *in vitro*. Compared to uncoated IOLs, the antibody coating inhibited LEC migration and adhesion. TGF-β inhibition by the monoclonal anti-TGF-β2 antibody CAT-152 was described by Wormstone et al. (2004). Human capsular bags (*ex vivo*) were incubated in medium with increasing levels of TGF-β2, with and without CAT-152. They concluded that addition of TGF-β2 to the medium resulted in an enhanced transdifferentiation of LECs and contraction of the capsular bag.The addition of CAT-152 completely inhibited the effects of TGF-β2. The agent pirfenidone was found to have anti-fibrotic properties by reducing proliferation and migration of LEC by inhibition of TGF-β2 signaling in a human LEC line *in vitro* (Yang et al., 2013). Pirfenidone inhibits the expression of Smads in TGF-β2 signaling (Smad2, Smad3, and Smad4), and it prevents the accumulation and translocation of these Smads to the nuclei of the LECs. For the transdifferentiation of LECs through TGF-β stimulation, activation of Rho GTPase is needed (Cho and Yoo, 2007). The statin lovastatin was found to inhibit these TGF-β-induced LEC changes through the blockage of Rho activity (Urakami et al., 2012). H-7, a broad spectrum serine/theonine kinase inhibitor, also has inhibitory effects on Rho kinase and was reported to prevent PCO formation and wrinkling of the lens capsule in human lens capsular bags *ex vivo* (Sureshkumar et al., 2012).

Interference with TGF-β signaling seems promising, as the TGF-β pathway seems to have the highest impact level on PCO development compared to other growth factors (de Iongh et al., 2005). However, influencing cellular signaling does not only affect the LECs but will definitely also affect other ocular cells. Unfortunately, recent research only describes *in vitro* and *ex vivo* experiments, which lack details on side effects to other ocular tissues. It has been known that TGF-β signaling is also important in corneal endothelium behavior after injury (Saika, 2004). Therefore, more research is required to determine all ocular effects of interference with TGF-β signaling in the lens.

Interfering with signaling of other growth factors

Awasthi et al. (2008) have used the FGF pathway to interfere with PCO formation by proteasome inhibition. Proteasomes are intracellular protein complexes with the main function

to degrade proteins which are damaged or redundant. Inhibition of proteasomes was found to downregulate MMP activity (Awasthi and Wagner, 2006), and MMP-2 is also responsible for FGF-2 release (Tholozan et al., 2007). In subsection 2.3 (page 47-48) the use of MMP inhibition in PCO prevention is discussed further. Wertheimer et al. (2013, 2014) recently tested the EGF receptor inhibitor erlotinib. Erlotinib inhibited LEC proliferation and migration and was found to reduce the expression of EGF and TGF-β2 in human LECs *in vitro*. Furthermore, no negative side effects to other ocular cell types were found, making erlotinib a potential agent for PCO prevention. These studies in growth factor signaling pathways provide indications for potential targets in PCO prevention. However, the study of Ivengar et al. (2009) already showed that multiple growth factors are responsible for PCO formation. This again demonstrates that the complexity of signaling systems and mutual involvements makes it difficult to find a solution in PCO prevention by targeting only one growth factor.

Interfering with integrin signaling

Integrin antagonists were found to inhibit PCO formation in rabbit eyes (Inan et al., 2001b, Kim et al., 2002, Nishi et al., 1997). In a study focusing on the disintegrin salmosin, it was found that LEC migration and proliferation could be inhibited by targeting integrins (Kim et al., 2002). Disintegrins interfere with the β-subunit of integrins and salmosin contains different recognition sites for integrins, including the RGD (arginine-glycine-aspartic acid) sequence. The effectiveness of RGD peptide in PCO prevention has been tested by Nishi et al. (1997). They found a slight inhibition of the migration of LECs in rabbit eyes by sustained-release of RGD peptide using a polylactic acid disk. However, when the RGD peptide release was combined with EDTA administration (subsection 2.3 page 47-48), a significant inhibition in PCO formation was described (Inan et al., 2001b, Nishi et al., 1997).

Since integrins can influence LEC behavior via a variety of pathways and growth factor receptors, interfering with integrin signaling probably will target more than one signaling pathway. Furthermore, integrins were found to be key activators for TGF-β signaling. So blocking integrins for PCO prevention provides a more holistic approach to influencing LEC behavior and could overcome the problems that arise when only one specific growth factor or pathway is manipulated.

Interfering with extracellular matrix remodeling

As mentioned in paragraph 2.2 (page 34), MMP-2 and MMP-9 are expressed in reaction to TGF-β release and they are involved in EMT-related matrix contraction (Wormstone

et al., 2002). Inhibition of MMP activity via proteasome inhibition decreased LEC migration and PCO formation in human capsular bags *ex vivo* (Awasthi et al., 2008). Ethylenediaminetetraacetic acid (EDTA) is known from its use in chelation therapy, but was also found to inhibit MMP activity and disrupt junctional complexes in LECs (Hazra et al., 2012a, Nishi et al., 1997). Therapy with EDTA reduced the migration and proliferation of LECs in rabbit lenses (Fernandez et al., 2004, Hazra et al., 2012a, Inan et al., 2001b, Nishi et al., 1996). However, also problems with ocular inflammation and corneal edema were described (Fernandez et al., 2004, Inan et al., 2001b).

Secreted protein acidic and rich in cysteine (SPARC) regulates ECM-LEC interactions by activation of ECM proteins and MMP expression (Gotoh et al., 2007, Wederell and de Iongh, 2006). Blockage of SPARC in mice resulted in increased migration and proliferation of LECs after TGF-β2 administration, indicating that SPARC has an inhibitory effect on LEC migration and proliferation (Gotoh et al., 2007). Another agent interfering with MMP expression is caffeic acid phenethyl ester (CAPE) (Hwang et al., 2006). CAPE is an active component of Propolis, which is produced by honeybees in their hives. CAPE reduces PCO in rabbit lenses (Hepsen et al., 1997). Furthermore, MMP expression can be influenced by nobiletin. Nobiletin is a polymethoxyflavone derived from citrus species and was found to inhibit the production of MMP-9. In human LECs *in vitro*, nobiletin suppressed proliferation in a dose-dependent manner (Miyata et al., 2013).

As previously reported, MMP expression can be influenced via different mechanisms and has also been associated to FGF-2 signaling, which consecutively has been associated to TGF-β2 signaling. MMP inhibition could therefore be a potential strategy in targeting multiple pathways for PCO prevention. However, MMPs were also found to be widely involved in corneal healing and neovascularization (Sivak and Fini, 2002). Accordingly, some studies using EDTA in rabbit eyes reported corneal edema (Fernandez et al., 2004, Inan et al., 2001b). One study has shown that the cell morphology of the corneal epithelium did not change after EDTA administration, however, they did describe mild corneal edema in the first three days post surgery (Hazra et al., 2012a). Thus, more *in vivo* experiments are required to determine if MMP inhibition can be used safely.

Interfering with epigenetic mechanisms

By interfering with epigenetic mechanisms, gene expression is changed via interference with DNA or RNA. Zebularine is a new DNA methyltransferase inhibitor which has the potential to inhibit LEC proliferation and migration *in vitro* (Zhou et al., 2012). This potential

is expressed by inhibition of TGF-β2-induced αSMA expression in LECs via a methyl-DNA binding protein responsible for myofibroblast differentiation, named Methyl CpG binding protein 2 (MeCP2) (Zhou et al., 2011). HDAC-mediated epigenetic mechanisms are upregulated in TGF-β2-induced EMT in LECs (paragraph 2.2 page 38), and the HDAC inhibitors Trichostatin A and Vorinostat were recently evaluated for the inhibition of EMT in LECs (Chen et al., 2013, Xie et al., 2014). Chen et al. (2013) found that Trichostatin A inhibited LEC proliferation and migration in two human LEC-lines *in vitro*. Another study by Xie et al. (2014) showed that Trichostatin A and Vorinostat inhibited TGF-β2-induced αSMA expression in human and porcine lens capsules *ex vivo* in a dose-dependent way.

Interfering with epigenetic mechanisms for PCO prevention has only recently been investigated and many additional factors, such as side effects to other ocular tissues, have yet to be assessed.

Non-pharmacological measures

Some non-pharmacological methods have been evaluated for the prevention of PCO.

Gene therapy

Gene therapy can play a role in the prevention of PCO by interfering in the process of TGF-β signaling. By changing the composition of the cellular components which are involved in the signaling, the process of PCO formation can be influenced. Inhibition of snail, an early-response gene of TGF-β signaling (see subsection 2.2 page 34), by a PI3K inhibitor was found to block the EMT reaction in LECs *in vitro* (Cho et al., 2007). Li et al. (2013a) investigated the effect of small interference RNA (siRNA) targeting of snail on TGF-β2 signaling in human LECs. They found an inhibitory effect of the siRNA treatment on the release of EMT markers like αSMA and vimentin. In a study of Zheng et al. (2012), siRNAs were used to target the type II TGF-β receptor of LECs *in vitro*. This transfection of the type II TGF-β receptor in LECs prevented the transdifferentiation and migration of LECs and additionally prohibits the release of the myofibroblast markers αSMA and fibronectin.

In suicide gene therapy, a transfected gene induces apoptosis. In studies into the effectiveness of retro/adenovirus-mediated suicide gene therapy it was reported that these therapies can target LECs *in vitro* (Couderc et al., 1999, Malecaze et al., 1999). In the *in vivo* model with rabbit lenses, only adenovirus-mediated therapy could prevent PCO. In a study of Yang et al. (2012), lentivirus-mediated suicide gene therapy targeting human LECs was found to inhibit PCO in residual LECs *in vitro*.

The use of gene therapy is still controversial, above all on ethical grounds but also the general mechanisms behind the therapy have to be improved since e.g. virus-mediated suicide gene therapy or introduction of siRNAs can induce an undesirable immune response (Borras et al., 1996). Thus, the effectiveness of gene therapy in the lens is highly dependent on the immune response in the eye. Furthermore, this response has already been triggered by performing cataract surgery as such. This indicates that more *in vivo* experiments with gene therapy are needed to evaluate its effectiveness and side effects in PCO prevention.

Osmotic effective solutions

Another mechanism of interest is prevention of PCO by the induction of osmotic changes in LECs (Crowston et al., 2004, Duncan et al., 2007, Fernandez et al., 2004, Kim et al., 2007, Rabsilber et al., 2007, Rekas et al., 2013). Hypo-osmotic distilled water causes swelling of LECs and was found to subsequently destroy the cell membrane in LECs derived from an anterior capsule *in vitro* (Crowston et al., 2004). Since this mechanism also applies to the corneal endothelium, hypo-osmotic solutions for therapeutic purposes need to be administered in the capsular bag only or the anterior chamber needs to be rinsed carefully. Rabsilber et al. (2007) performed sealed-capsule irrigation with distilled water in one eye of patients undergoing cataract surgery for both eyes, but they did not find a significant difference between the PCO rates in the treated versus the control eyes. However, in a recent study, Rekas et al. (2013) have described a significant reduction of PCO rates in long term follow up of eyes treated with sealed-capsule irrigation of deionized distilled water. They treated the capsules for three minutes, compared to two minutes of Rabsilber. Furthermore, in the study of Rekas, patients were randomly assigned to a treatment or control group, while Rabsilber made a comparison between one treated and one untreated eye within the same patient. Contrastingly, Duncan et al. (2007) found that human LECs, *in vitro* and *ex vivo* on human donor capsular bags, react more to hyper-osmotic stress than to hypo-osmotic stress. LECs were exposed to different agents, including distilled water and a hyper-osmotic NaCl solution. There were significantly less LECs in both groups compared to the control group, but LECs treated with hyper-osmotic NaCl had a much higher apoptosis response than LECs treated with distilled water. In conclusion, the effectiveness of osmotic treatment of the LECs is not yet clear and hypo- or hyper-osmotic solutions have to be administrated carefully. However, manipulation of osmosis could be a possible system of PCO prevention, also in combination with other treatments.

Regeneration of the lens

Another altogether different path for the prevention of PCO could be lens regeneration. When a tissue endures trauma, the subsequent response will be either fibrosis or regeneration. The steps that determine which pathway will be followed are still unknown. After cataract surgery, the LECs experience trauma and normally enter the process of PCO with irregular growth of cells. However, LECs were also found to have regenerative properties and were capable of forming a capsule-like structure (Saika et al., 2001). Furthermore, when lens fibers are extracted without insertion of an IOL, regrowth of lensmaterial will form an irregular lens (Kessler, 1975). Yet if the lens fibers are exposed to a scaffold they are able to regenerate in a more natural way (Gwon and Gruber, 2010). This is because the scaffold provides an environment which mimics the normal native environment of the lens fibers, making them able to regenerate. If it were possible to regenerate the lens instead of treating the complications of fibrosis induced by cataract surgery, the problem of PCO would also be resolved.

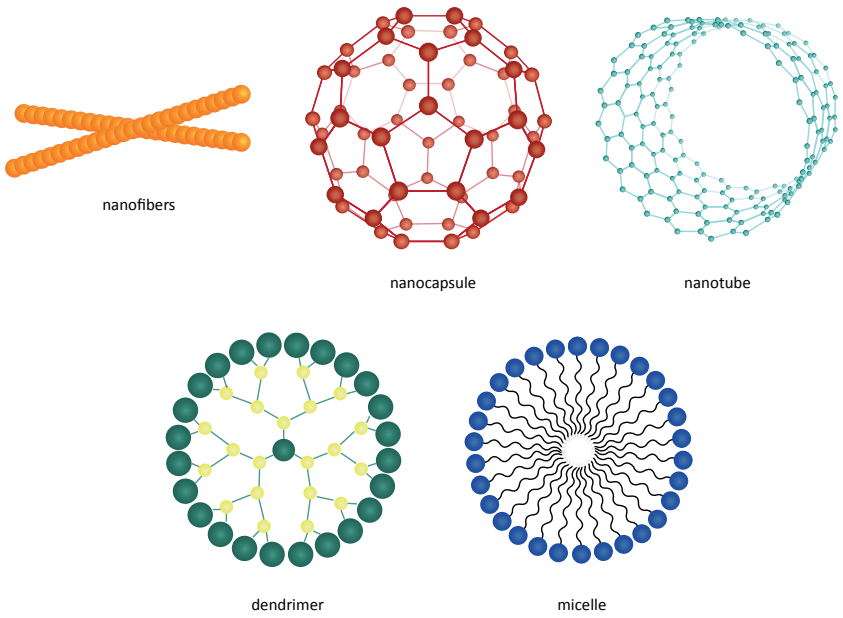


FIGURE 5. *Schematic representation of nanostructures which can be used in nanomedicine. The spheres represent the molecules and the lines show their chemical interaction which creates the specific appearance of each structure.*

2.4 Potential of nanomaterials

Nanomedicine involves the medical application of nanotechnology and is of growing importance in all fields of medicine, including ophthalmology (Etheridge et al., 2013). Nanotechnology makes use of nanomaterials, which are structures between 1 and 1000 nm in size, but usually smaller than 100 nm (Kreyling et al., 2010). Nanomaterials can consist of a broad variety of materials of either organic or inorganic origin occurring in various organizational constructs, which can be reversibly or irreversibly formed (Weissig et al., 2014). The morphology of the nanostructures can range from spheres, fibers, capsules, tubes, dendrimers, micelles to irregularly shaped particles (Euliss et al., 2006, Gentleman and Chan, 2009). Some examples of nanostructures are given in Figure 5. These structures could e.g. be used for targeting molecular interactions, drug delivery, and tissue engineering (Goldberg et al., 2007). Nanoparticles (in medicine) occur in various dispersed phases, like hydrogels, emulsions, and suspensions, depending on the dispersion medium and the physicochemical characteristics of the nanoparticle (Etheridge et al., 2013). For many medical applications hydrogels are preferred due to their permeable and flexible nature, resembling natural tissues. Figure 6 shows the surface of a hydrogel, indicating the great level of structural details. Often these gels consist of hydrophilic nanofibers which are prepared by processes such as electrospinning (Loh et al., 2010), interfacial polymerization (Oh et al., 2008), or self-assembly (Ikonen et al., 2013). Depending on their chemical properties, nanofibers allow for functionalization with small chemical entities, such as pharmaceuticals or synthetic mimics of cellular interaction sites, or even larger structures, such as proteins (like growth factors) thus mimicking natural tissues even further (Cui et al., 2010).

Nanomaterials in ophthalmology

In ophthalmology nanomaterials are currently examined for the use in retinal implants (Chen et al., 2011, Pritchard et al., 2010, Redenti et al., 2009), the development of artificial corneas (Bakhshandeh et al., 2011, Ionescu et al., 2011), the treatment of ocular surface injuries by stem cell therapy (Zajicova et al., 2010), and ocular drug delivery systems (Ellis-Behnke and Jonas, 2011, Zarbin et al., 2010). Different types of ocular drug delivery have been investigated, including topical administration (Hattori et al., 2012, Li et al., 2013b), intraocular, or subconjunctival administration (Guha et al., 2013, Kaiser et al., 2013, Pei et al., 2013, Weidmann et al., 2008) and by a coating on IOLs (Huang et al., 2013). Drug

delivery by nanomaterials improves the bioavailability of drugs and can prevent tissue toxicity due to sustained local release.

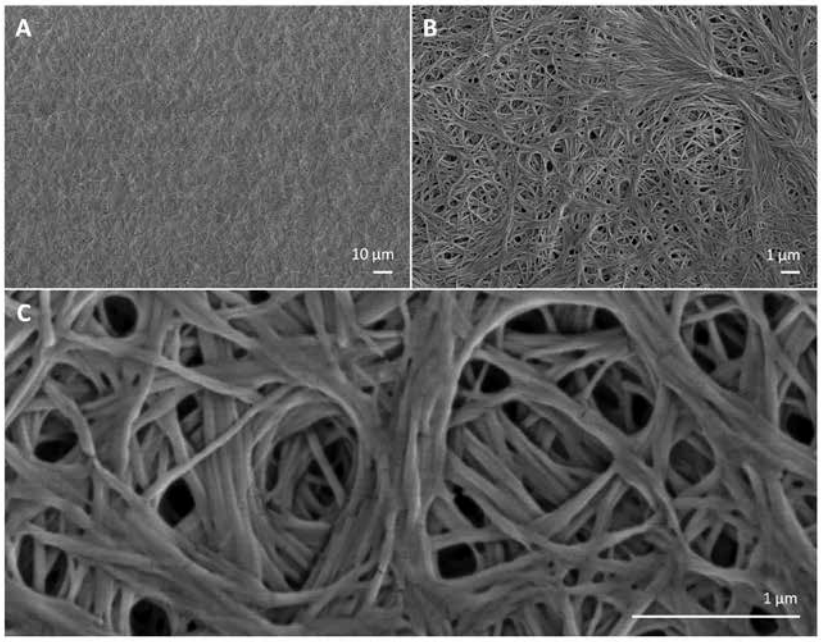


FIGURE 6. *Scanning electron microscopic images from a hydrogel in three magnifications (images A, B and C). The surface topography shows the high level of structural details of the hydrogel.*

Nanomaterials to prevent posterior capsular opacification

To date, few studies have examined nanomaterials for the prevention of PCO. There are different approaches to prevent PCO by the use of nanomedicine. First of all, the nanomaterials themselves can be used to target LECs for the prevention of PCO. Wang et al. (2013a) performed a study in which they used zinc oxide nanoparticles in combination with UVB irradiation to prevent PCO. They tested these nanoparticles for the biological inhibition of calcium transport in LECs *in vitro* and found that zinc oxide nanoparticles and UVB irradiation have a synergistic effect on the inhibition of LEC proliferation.

Secondly, nanomaterials can be used as a drug delivery system. As mentioned before (subsection 2.3 page 44), Huang et al. (2013) have examined the sustained-release of 5-fluorouracil (5-FU) from nanoparticles using surface modification of IOLs. They found a decrease in proliferation and an induction of apoptosis in human LECs and in rabbit

lens capsules. Furthermore, post surgical inflammation was reduced, probably due to the sustained-release of 5-FU. In a study of Guha et al. (2013), nanoparticles loaded with the cytotoxic agent doxorubicin were analyzed for the prevention of PCO. They observed a decrease in PCO formation after subconjunctival sustained-release of doxorubicin by the nanoparticles in rabbits *in vivo*. In another recent study (Zhang et al., 2013) a lipid nanoparticle was designed for delivery of the drug genistein, which was found to inhibit PCO formation by tyrosine kinase inhibition (Walker et al., 2007). The nanoparticle provided a controlled release of genistein for 72 hours and inhibited LEC growth during this period. As shown by these recent studies, nanomaterials can be drug delivery systems with great potential for PCO prevention.

Adequately tailored nanomaterials (e.g. nanofibers), applied as a layer on the inner surface of the capsular bag, may have the potential to affect LEC function and morphology (Cui et al., 2010). This layer could be a coating on IOLs, but also hydrogels which are injected in the capsular bag are a possible solution. The tailoring of the coating or hydrogel could consist of bound peptides or drugs to provide an environment that effectively prevents PCO formation. This methodology might also prove successful in preventing PCO in injectable accommodating lenses (Koopmans et al., 2011). Nowadays, the main problem in the development of injectable accommodating lenses is the formation of PCO (van Kooten et al., 2006). Accommodating lenses require an intact lens capsule as a surrounding for the injected polymer and to provide accommodation of the new refilled lens. Functionalized hydrogels could form a layer between the inserted lens refilling material and the surface of the capsular bag thus preventing material induced PCO formation.

2.5 Future perspectives

In this review we described recent progress in research for the prevention of PCO and the development of new techniques and therapeutic strategies in PCO prevention. So far, major steps were made with the recognition of the signaling pathways concerning TGF-β and other growth factors involved in the PCO process. Yet effects of treatment are difficult to compare because quantification of treatment effects is not standardized and none of the methods interfering with these biological processes are clinically used. The only methods for PCO prevention that seem to work in the clinic are methods making use

of mechanical prevention such as the sharp edged IOL. Results from studies targeting cellular signaling are encouraging for the future use of the corresponding agents in PCO prevention. Moreover, the field of nanotechnology is a welcome addition to the currently known therapeutic options for prevention of PCO.

It is still not known what would be the best way to target LECs for PCO prevention. Numerous studies have attempted to prevent PCO using a wide range of approaches, for example by pharmacological inhibition of proliferation, induction of LEC apoptosis, or interference with biological processes, but none succeeded in complete PCO prevention. It seems that targeting one specific pathway is not enough. This hypothesis is supported by the complexity of the signaling systems involved in PCO and by the extent that they influence one another and raises the question if targeting one single growth factor will ever result in complete prevention of PCO formation. On the other hand, it still could be possible that providing a proper environment for the residual cells in the lens capsule, resulting in maintenance of the epithelial phenotype, is a better approach to PCO prevention after cataract surgery. In this approach, nanomaterials could be used to mimic the natural environment of the cells. The theory of providing a natural environment for cells can also be linked to the possibilities of lens regeneration. When lens fibers are exposed to their normal environment by a scaffold, they have the ability to grow in a structured way, resulting in a transparent lens (section 2.3 page 51).

Furthermore, it is debatable if complete PCO prevention is required or if a decrease of PCO formation is sufficient to improve clinical visual outcomes. In a recent study by Spalton et al. (2014) it was described that total LEC destruction could lead to problems with IOL fixation in the capsular bag. This indicates that it might be preferable to only partially prevent LEC growth or that IOL fixation needs to be adjusted when total PCO prevention becomes possible.

In future solutions we expect that the biological processes underlying the development of PCO will be the target for PCO prevention. We also expect that combinations of targets in PCO prevention will be more effective than single targets. The integration of nanomaterials in potential systems may be of benefit as this opens a path to preserving the epithelial phenotype in LEC circumventing PCO altogether. We envisage that combining the knowledge of pharmacological and biological processes with the addition of nanotechnological advances may eventually result in the prevention of PCO.

2.6 Summary

PCO is a common complication after cataract surgery. The development of PCO consists of a combination of the processes of proliferation, migration, and transdifferentiation of the residual cells on the lens capsule. Usually, PCO in the central visual axis is treated with a Nd:YAG laser capsulotomy. Since this treatment entails risks on complications to other structures of the eye, the need for PCO prevention becomes increasingly important. During the past decades, various forms of prevention have been examined, including general measures during surgery (like surgical techniques, IOL materials and designs), pharmacological prevention, and the prevention of PCO by interfering with the biological processes of EMT in LECs. To date, there is little quantified data and the only method that seems effective in the clinic is the implantation of IOLS with sharp edged optics to mechanically prevent PCO formation.

Since the recognition of the process of EMT, biological processes involved in the formation of PCO became known, and many growth factors were found to be involved in the development of PCO. The growth factor TGF-β plays the most significant role as it is involved in the process of cell function regulation in LECs. Inappropriate TGF-β signaling causes PCO through different signaling pathways, including TGF-β/Smad, Rho, PI3K/Akt, MAPK, ERK1/2, and Notch. Other pathways of signaling by cytokines, receptors, and different growth factors are also described to be involved in PCO development, including FGF, HGF, EGF, IGF, PDGF, CTGF, BMP, Wnt, TNF-α, integrins, CD44/hyaluronan, and HDACs. Several studies have evaluated the interference with the specific steps of these pathways to target LEC signaling and prevent PCO formation. Other approaches of PCO prevention include gene therapy, affecting LECs by osmotic changes, and regeneration of the lens fibers instead of replacing the natural lens by an IOL. Furthermore, nanomaterials could play a role in the prevention of PCO. Nanotechnology gains an increasing share in current medicine and has recently been examined for different ocular applications. However, only a few studies evaluated the use of nanomaterials for PCO prevention. We expect that in future experiments combinations of pharmacological treatment of the underlying biological processes and the use of nanomedicine could be promising for the prevention of PCO.

Acknowledgements

This work was supported by the European Fund for Regional Development, Samenwerkingsverband Noord-Nederland, and NanoNextNL, a micro and nanotechnology consortium of the Government of the Netherlands and 130 partners. The authors want to thank Annemarie Tuin for providing the scanning electron microscopic images.

References

Acharya P.S., Majumdar S., Jacob M., Hayden J., Mrass P., Weninger W., Assoian R.K., Pure E., 2008. Fibroblast migration is mediated by CD44-dependent TGF beta activation. J. Cell. Sci. 121, 1393-1402.

Apple D.J., Solomon K.D., Tetz M.R., Assia E.I., Holland E.Y., Legler U.F., Tsai J.C., Castaneda V.E., Hoggatt J.P., Kostick A.M., 1992. Posterior capsule opacification. Surv. Ophthalmol. 37, 73-116.

Aslam T.M., Devlin H., Dhillon B., 2003. Use of Nd:YAG laser capsulotomy. Surv. Ophthalmol. 48, 594-612.

Awasthi N., Guo S., Wagner B.J., 2009. Posterior capsular opacification: a problem reduced but not yet eradicated. Arch. Ophthalmol. 127, 555-562.

Awasthi N., Wagner B.J., 2006. Suppression of human lens epithelial cell proliferation by proteasome inhibition, a potential defense against posterior capsular opacification. Invest. Ophthalmol. Vis. Sci. 47, 4482-4489.

Awasthi N., Wang-Su S.T., Wagner B.J., 2008. Downregulation of MMP-2 and -9 by proteasome inhibition: a possible mechanism to decrease LEC migration and prevent posterior capsular opacification. Invest. Ophthalmol. Vis. Sci. 49, 1998-2003.

Bakhshandeh H., Soleimani M., Hosseini S.S., Hashemi H., Shabani I., Shafiee A., Nejad A.H., Erfan M., Dinarvand R., Atyabi F., 2011. Poly (epsilon-caprolactone) nanofibrous ring surrounding a polyvinyl alcohol hydrogel for the development of a biocompatible two-part artificial cornea. Int. J. Nanomedicine 6, 1509-1515.

Bao X.L., Song H., Chen Z., Tang X., 2012. Wnt3a promotes epithelial-mesenchymal transition, migration, and proliferation of lens epithelial cells. Mol. Vis. 18, 1983-1990.

Behar-Cohen F.F., David T., D'Hermies F., Pouliquen Y.M., Buechler Y., Nova M.P., Houston L.L., Courtois Y., 1995. In vivo inhibition of lens regrowth by fibroblast growth factor 2-saporin. Invest. Ophthalmol. Vis. Sci. 36, 2434-2448.

Birinci H., Kuruoglu S., Oge I., Oge F., Acar E., 1999. Effect of intraocular lens and anterior capsule opening type on posterior capsule opacification. J. Cataract Refract. Surg. 25, 1140-1146.

Borras T., Tamm E.R., Zigler J.S.,Jr, 1996. Ocular adenovirus gene transfer varies in efficiency and inflammatory response. Invest. Ophthalmol. Vis. Sci. 37, 1282-1293.

Bretton R.H., Swearingen A., Kash R.L., Cooley R., 1999. Use of a polylysine-saporin conjugate to prevent posterior capsule opacification. J. Cataract Refract. Surg. 25, 921-929.

Cammas L., Wolfe J., Choi S.Y., Dedhar S., Beggs H.E., 2012. Integrin-linked kinase deletion in the

developing lens leads to capsule rupture, impaired fiber migration and non-apoptotic epithelial cell death. Invest. Ophthalmol. Vis. Sci. 53, 3067-3081.

Chandler H.L., Barden C.A., Lu P., Kusewitt D.F., Colitz C.M., 2007. Prevention of posterior capsular opacification through cyclooxygenase-2 inhibition. Mol. Vis. 13, 677-691.

Chandler H.L., Haeussler D.J.,Jr, Gemensky-Metzler A.J., Wilkie D.A., Lutz E.A., 2012. Induction of posterior capsule opacification by hyaluronic acid in an ex vivo model. Invest. Ophthalmol. Vis. Sci. 53, 1835-1845.

Chen H., Fan X., Xia J., Chen P., Zhou X., Huang J., Yu J., Gu P., 2011. Electrospun chitosan-graft-poly (varepsilon-caprolactone)/poly (varepsilon-caprolactone) nanofibrous scaffolds for retinal tissue engineering. Int. J. Nanomedicine 6, 453-461.

Chen X., Xiao W., Chen W., Luo L., Ye S., Liu Y., 2013. The epigenetic modifier trichostatin A, a histone deacetylase inhibitor, suppresses proliferation and epithelial-mesenchymal transition of lens epithelial cells. Cell. Death Dis. 4, e884.

Chen X., Ye S., Xiao W., Wang W., Luo L., Liu Y., 2014. ERK1/2 pathway mediates epithelial-mesenchymal transition by cross-interacting with TGFbeta/Smad and Jagged/Notch signaling pathways in lens epithelial cells. Int. J. Mol. Med. 33, 1664-1670.

Cho H.J., Baek K.E., Saika S., Jeong M.J., Yoo J., 2007. Snail is required for transforming growth factor-beta-induced epithelial-mesenchymal transition by activating PI3 kinase/Akt signal pathway. Biochem. Biophys. Res. Commun. 353, 337-343.

Cho H.J., Yoo J., 2007. Rho activation is required for transforming growth factor-beta-induced epithelial-mesenchymal transition in lens epithelial cells. Cell Biol. Int. 31, 1225-1230.

Choi J., Park S.Y., Joo C.K., 2004. Hepatocyte growth factor induces proliferation of lens epithelial cells through activation of ERK1/2 and JNK/SAPK. Invest. Ophthalmol. Vis. Sci. 45, 2696-2704.

Choi J., Park S.Y., Joo C.K., 2007. Transforming growth factor-beta1 represses E-cadherin production via slug expression in lens epithelial cells. Invest. Ophthalmol. Vis. Sci. 48, 2708-2718.

Chong C.C., Stump R.J., Lovicu F.J., McAvoy J.W., 2009. TGFbeta promotes Wnt expression during cataract development. Exp. Eye Res. 88, 307-313.

Clark D.S., Emery J.M., Munsell M.F., 1998. Inhibition of posterior capsule opacification with an immunotoxin specific for lens epithelial cells: 24 month clinical results. J. Cataract Refract. Surg. 24, 1614-1620.

Cortina P., Gomez-Lechon M.J., Navea A., Menezo J.L., Terencio M.C., Diaz-Llopis M., 1997. Diclofenac sodium and cyclosporin A inhibit human lens epithelial cell proliferation in culture. Graefes Arch. Clin. Exp. Ophthalmol. 235, 180-185.

Couderc B.C., de Neuville S., Douin-Echinard V., Serres B., Manenti S., Darbon J.M., Malecaze F., 1999. Retrovirus-mediated transfer of a suicide gene into lens epithelial cells in vitro and in an experimental model of posterior capsule opacification. Curr. Eye Res. 19, 472-482.

Crowston J.G., Healey P.R., Hopley C., Neilson G., Milverton E.J., Maloof A., 2004. Water-mediated lysis of lens epithelial cells attached to lens capsule. J. Cataract Refract. Surg. 30, 1102-1106.

Cui H., Webber M.J., Stupp S.I., 2010. Self-assembly of peptide amphiphiles: from molecules to nanostructures to biomaterials. Biopolymers 94, 1-18.

Davis J.L., Yi N.Y., Salmon J.H., Charlton A.N., Colitz C.M., Gilger B.C., 2012. Sustained-Release Celecoxib from Incubated Acrylic Intraocular Lenses Suppresses Lens Epithelial Cell Growth in an Ex Vivo Model of Posterior Capsule Opacity. J. Ocul. Pharmacol. Ther. 28, 359-368.

Dawes L.J., Duncan G., Wormstone I.M., 2013. Age-related differences in signaling efficiency of human lens cells underpin differential wound healing response rates following cataract surgery. Invest. Ophthalmol. Vis. Sci. 54, 333-342.

Dawes L.J., Elliott R.M., Reddan J.R., Wormstone Y.M., Wormstone I.M., 2007. Oligonucleotide microarray analysis of human lens epithelial cells: TGFbeta regulated gene expression. Mol. Vis. 13, 1181-1197.

de Iongh R.U., Wederell E., Lovicu F.J., McAvoy J.W., 2005. Transforming growth factor-beta-induced epithelial-mesenchymal transition in the lens: a model for cataract formation. Cells Tissues Organs 179, 43-55.

Duncan G., Wang L., Neilson G.J., Wormstone I.M., 2007. Lens cell survival after exposure to stress in the closed capsular bag. Invest. Ophthalmol. Vis. Sci. 48, 2701-2707.

Duncan G., Wormstone I.M., Liu C.S., Marcantonio J.M., Davies P.D., 1997. Thapsigargin-coated intraocular lenses inhibit human lens cell growth. Nat. Med. 3, 1026-1028.

Eldred J.A., Dawes L.J., Wormstone I.M., 2011. The lens as a model for fibrotic disease. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 366, 1301-1319.

Ellis-Behnke R., Jonas J.B., 2011. Redefining tissue engineering for nanomedicine in ophthalmology. Acta Ophthalmol. 89, e108-14.

Etheridge M.L., Campbell S.A., Erdman A.G., Haynes C.L., Wolf S.M., McCullough J., 2013. The big picture on nanomedicine: the state of investigational and approved nanomedicine products. Nanomedicine 9, 1-14.

Euliss L.E., DuPont J.A., Gratton S., DeSimone J., 2006. Imparting size, shape, and composition control of materials for nanomedicine. Chem. Soc. Rev. 35, 1095-1104.

Fernandez V., Fragoso M.A., Billotte C., Lamar P., Orozco M.A., Dubovy S., Willcox M., Parel J.M., 2004. Efficacy of various drugs in the prevention of posterior capsule opacification: experimental study of rabbit eyes. J. Cataract Refract. Surg. 30, 2598-2605.

Findl O., Buehl W., Bauer P., Sycha T., 2010. Interventions for preventing posterior capsule opacification. Cochrane Database Syst. Rev. (2), CD003738.

Funahashi A., Morohashi M., Kitano H., Tanimura N., 2003. CellDesigner: a process diagram editor for gene-regulatory and biochemical networks. Biosilico 1, 159-162.

Ge J., Wand M., Chiang R., Paranhos A., Shields M.B., 2000. Long-term effect of Nd:YAG laser posterior capsulotomy on intraocular pressure. Arch. Ophthalmol. 118, 1334-1337.

Geissler F.T., Li D.W., James E.R., 2001. Inhibition of lens epithelial cell growth by induction of apoptosis: potential for prevention of posterior capsule opacification. J. Ocul. Pharmacol. Ther. 17, 587-596.

Gentleman D.J., Chan W.C., 2009. A systematic nomenclature for codifying engineered nanostructures. Small 5, 426-431.

Georgopoulos M., Menapace R., Findl O., Petternel V., Kiss B., Rainer G., 2003. After-cataract in adults with primary posterior capsulorhexis: comparison of hydrogel and silicone intraocular lenses with round edges after 2 years. J. Cataract Refract. Surg. 29, 955-960.

Goldberg M., Langer R., Jia X., 2007. Nanostructured materials for applications in drug delivery and tissue engineering. J. Biomater. Sci. Polym. Ed. 18, 241-268.

Gotoh N., Perdue N.R., Matsushima H., Sage E.H., Yan Q., Clark J.I., 2007. An in vitro model of posterior capsular opacity: SPARC and TGF-beta2 minimize epithelial-to-mesenchymal transition in lens epithelium. Invest. Ophthalmol. Vis. Sci. 48, 4679-4687.

Guha R., Chowdhury S., Palui H., Mishra A., Basak S., Mandal T.K., Hazra S., Konar A., 2013. Doxorubicin-loaded MePEG-PCL nanoparticles for prevention of posterior capsular opacification. Nanomedicine (Lond) 8, 1415-1428.

Gwon A., Gruber L., 2010. Engineering the crystalline lens with a biodegradable or non-degradable scaffold. Exp. Eye Res. 91, 220-228.

Hattori M., Shimizu K., Katsumura K., Oku H., Sano Y., Matsumoto K., Yamaguchi Y., Ikeda T., 2012. Effects of all-trans retinoic acid nanoparticles on corneal epithelial wound healing. Graefes Arch. Clin. Exp. Ophthalmol. 250, 557-563.

Hazra S., Guha R., Jongkey G., Palui H., Mishra A., Vemuganti G.K., Basak S.K., Mandal T.K., Konar A., 2012a. Modulation of matrix metalloproteinase activity by EDTA prevents posterior capsular opacification. Mol. Vis. 18, 1701-1711.

Hazra S., Palui H., Vemuganti G.K., 2012b. Comparison of design of intraocular lens versus the material for PCO prevention. Int. J. Ophthalmol. 5, 59-63.

Hepsen I.F., Bayramlar H., Gultek A., Ozen S., Tilgen F., Evereklioglu C., 1997. Caffeic acid phenethyl ester to inhibit posterior capsule opacification in rabbits. J. Cataract Refract. Surg. 23, 1572-1576.

Hollick E.J., Spalton D.J., Ursell P.G., Meacock W.R., Barman S.A., Boyce J.F., 2000. Posterior capsular opacification with hydrogel, polymethylmethacrylate, and silicone intraocular lenses: two-year results of a randomized prospective trial. Am. J. Ophthalmol. 129, 577-584.

Huang W.R., Fan X.X., Tang X., 2011. SiRNA targeting EGFR effectively prevents posterior capsular opacification after cataract surgery. Mol. Vis. 17, 2349-2355.

Huang X., Wang Y., Cai J.P., Ma X.Y., Li Y., Cheng J.W., Wei R.L., 2013. Sustained release of 5-fluorouracil from chitosan nanoparticles surface modified intra ocular lens to prevent posterior capsule opacification: an in vitro and in vivo study. J. Ocul. Pharmacol. Ther. 29, 208-215.

Hwang H.J., Park H.J., Chung H.J., Min H.Y., Park E.J., Hong J.Y., Lee S.K., 2006. Inhibitory effects of caffeic acid phenethyl ester on cancer cell metastasis mediated by the down-regulation of matrix metalloproteinase expression in human HT1080 fibrosarcoma cells. J. Nutr. Biochem. 17, 356-362.

Ibaraki N., Chen S.C., Lin L.R., Okamoto H., Pipas J.M., Reddy V.N., 1998. Human lens epithelial cell line. Exp. Eye Res. 67, 577-585.

Ikonen L., Kerkela E., Metselaar G., Stuart M.C., de Jong M.R., Aalto-Setala K., 2013. 2D and 3D self-assembling nanofiber hydrogels for cardiomyocyte culture. Biomed. Res. Int. 2013, 285678.

Inan U.U., Ozturk F., Kaynak S., Ilker S.S., Ozer E., Guler C., 2001a. Prevention of posterior capsule opacification by retinoic acid and mitomycin. Graefes Arch. Clin. Exp. Ophthalmol. 239, 693-697.

Inan U.U., Ozturk F., Kaynak S., Kurt E., Emiroglu L., Ozer E., Ilker S.S., Guler C., 2001b. Prevention of posterior capsule opacification by intraoperative single-dose pharmacologic agents. J. Cataract Refract. Surg. 27, 1079-1087.

Ionescu A.M., Alaminos M., de la Cruz Cardona J., de Dios Garcia-Lopez Duran J., Gonzalez-Andrades M., Ghinea R., Campos A., Hita E., del Mar Perez M., 2011. Investigating a novel nanostructured fibrin-agarose biomaterial for human cornea tissue engineering: rheological properties. J. Mech. Behav. Biomed. Mater. 4, 1963-1973.

Ishibashi T., Hatae T., Inomata H., 1994. Collagen types in human posterior capsule opacification. J. Cataract Refract. Surg. 20, 643-646.

Iyengar L., Patkunanathan B., McAvoy J.W., Lovicu F.J., 2009. Growth factors involved in aqueous humour-induced lens cell proliferation. Growth Factors 27, 50-62.

Joussen A.M., Huppertz B., Koch H.R., Kernert N., Camphausen K., Schlosser K., Foerster A.M., Kruse F.E., Lappas A., Kirchhof B., 2001. Low-dose-rate ionizing irradiation for inhibition of secondary cataract formation. Int. J. Radiat. Oncol. Biol. Phys. 49, 817-825.

Kaiser J.M., Imai H., Haakenson J.K., Brucklacher R.M., Fox T.E., Shanmugavelandy S.S., Unrath K.A., Pedersen M.M., Dai P., Freeman W.M., Bronson S.K., Gardner T.W., Kester M., 2013. Nanoliposomal minocycline for ocular drug delivery. Nanomedicine 9, 130-140.

Kalluri R., Neilson E.G., 2003. Epithelial-mesenchymal transition and its implications for fibrosis. J. Clin. Invest. 112, 1776-1784.

Kardassis D., Murphy C., Fotsis T., Moustakas A., Stournaras C., 2009. Control of transforming growth factor beta signal transduction by small GTPases. FEBS J. 276, 2947-2965.

Kessler J., 1975. Lens refilling and regrowth of lens substance in the rabbit eye. Ann. Ophthalmol. 7, 1059-1062.

Kim J.T., Lee D.H., Chung K.H., Kang I.C., Kim D.S., Joo C.K., 2002. Inhibitory effects of salmosin, a disintegrin, on posterior capsular opacification in vitro and in vivo. Exp. Eye Res. 74, 585-594.

Kim S.Y., Kim J.H., Choi J.S., Joo C.K., 2007. Comparison of posterior capsule opacification in rabbits receiving either mitomycin-C or distilled water for sealed-capsule irrigation during cataract surgery. Clin. Experiment. Ophthalmol. 35, 755-758.

Koopmans S.A., Terwee T., Hanssen A., Martin H., Langner S., Stachs O., van Kooten T.G., 2014. Prevention of capsule opacification after accommodating lens refilling: pilot study of strategies evaluated in a monkey model. J. Cataract Refract. Surg. 40, 1521-1535.

Koopmans S.A., Terwee T., van Kooten T.G., 2011. Prevention of capsular opacification after accommodative lens refilling surgery in rabbits. Biomaterials 32, 5743-5755.

Kreyling W.G., Semmler-Behnke M., Chaudhry Q., 2010. A complementary definition of nanomaterial. Nano Today 5, 165-168.

Kubo E., Hasanova N., Fatma N., Sasaki H., Singh D.P., 2013. Elevated tropomyosin expression is associated with epithelial-mesenchymal transition of lens epithelial cells. J. Cell. Mol. Med. 17, 212-221.

Kugelberg M., Shafiei K., van der Ploeg I., Zetterstrom C., 2010. Intraocular lens as a drug delivery system for dexamethasone. Acta Ophthalmol. 88, 241-244.

Lee H., O'Meara S.J., O'Brien C., Kane R., 2007. The role of gremlin, a BMP antagonist, and epithelial-to-mesenchymal transition in proliferative vitreoretinopathy. Invest. Ophthalmol. Vis. Sci. 48, 4291-4299.

Leishman L., Werner L., Bodnar Z., Ollerton A., Michelson J., Schmutz M., Mamalis N., 2012. Prevention of capsular bag opacification with a modified hydrophilic acrylic disk-shaped intraocular lens. J. Cataract Refract. Surg. 38, 1664-1670.

Li H., Yuan X., Li J., Tang X., 2014. Implication of Smad2 and Smad3 in Transforming Growth Factor-beta-induced Posterior Capsular Opacification of Human Lens Epithelial Cells. Curr. Eye Res. 9, 1-12.

Li J., Tang X., Chen X., 2011. Comparative effects of TGF-beta2/Smad2 and TGF-beta2/Smad3 signaling pathways on proliferation, migration, and extracellular matrix production in a human lens cell line. Exp. Eye Res. 92, 173-179.

Li P., Jing J., Hu J., Li T., Sun Y., Guan H., 2013a. RNA Interference Targeting Snail Inhibits the Transforming Growth Factor beta 2-Induced Epithelial-Mesenchymal Transition in Human Lens Epithelial Cells. J. Ophthalmol. 2013, 869101.

Li X., Zhang Z., Chen H., 2013b. Development and evaluation of fast forming nano-composite hydrogel for ocular delivery of diclofenac. Int. J. Pharm. 448, 96-100.

Linnola R.J., Werner L., Pandey S.K., Escobar-Gomez M., Znoiko S.L., Apple D.J., 2000a. Adhesion of fibronectin, vitronectin, laminin, and collagen type IV to intraocular lens materials in pseudophakic human autopsy eyes. Part 1: histological sections. J. Cataract Refract. Surg. 26, 1792-1806.

Linnola R.J., Werner L., Pandey S.K., Escobar-Gomez M., Znoiko S.L., Apple D.J., 2000b. Adhesion of fibronectin, vitronectin, laminin, and collagen type IV to intraocular lens materials in pseudophakic human autopsy eyes. Part 2: explanted intraocular lenses. J. Cataract Refract. Surg. 26, 1807-1818.

Liu H., Feng G., Wu L., Fu S., Liu P., Yang W., Zhang X., 2010. The effects of rapamycin on lens epithelial cell proliferation, migration, and matrix formation: an in vitro study. Mol. Vis. 16, 1646-1653.

Liu H., Wu L., Fu S., Hou Y., Liu P., Cui H., Liu J., Xing L., Zhang X., 2009. Polylactide-glycoli acid and rapamycin coating intraocular lens prevent posterior capsular opacification in rabbit eyes. Graefes Arch. Clin. Exp. Ophthalmol. 247, 801-807.

Liu X., Cheng B., Zheng D., Liu Y., Liu Y., 2010. Role of anterior capsule polishing in residual lens epithelial cell proliferation. J. Cataract Refract. Surg. 36, 208-214.

Loh X.J., Peh P., Liao S., Sng C., Li J., 2010. Controlled drug release from biodegradable thermoresponsive physical hydrogel nanofibers. J. Control. Release 143, 175-182.

Lopez-Novoa J.M., Nieto M.A., 2009. Inflammation and EMT: an alliance towards organ fibrosis and cancer progression. EMBO Mol. Med. 1, 303-314.

Lovicu F.J., McAvoy J.W., 2001. FGF-induced lens cell proliferation and differentiation is dependent on MAPK (ERK1/2) signalling. Development 128, 5075-5084.

Lovicu F.J., McAvoy J.W., de Iongh R.U., 2011. Understanding the role of growth factors in embryonic development: insights from the lens. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 366, 1204-1218.

Lovicu F.J., Schulz M.W., Hales A.M., Vincent L.N., Overbeek P.A., Chamberlain C.G., McAvoy J.W., 2002. TGFbeta induces morphological and molecular changes similar to human anterior subcapsular cataract. Br. J. Ophthalmol. 86, 220-226.

Ma B., Kang Q., Qin L., Cui L., Pei C., 2014. TGF-beta2 induces transdifferentiation and fibrosis in human lens epithelial cells via regulating gremlin and CTGF. Biochem. Biophys. Res. Commun. 447, 689-695.

Maedel S., Hirnschall N., Chen Y.A., Findl O., 2013. Effect of heparin coating of a foldable intraocular lens on inflammation and capsular bag performance after cataract surgery. J. Cataract Refract. Surg. 39, 1810-1817.

Malecaze F., Couderc B., de Neuville S., Serres B., Mallet J., Douin-Echinard V., Manenti S., Revah F., Darbon J.M., 1999. Adenovirus-mediated suicide gene transduction: feasibility in lens epithelium and in prevention of posterior capsule opacification in rabbits. Hum. Gene Ther. 10, 2365-2372.

Maloof A., Neilson G., Milverton E.J., Pandey S.K., 2003. Selective and specific targeting of lens epithelial cells during cataract surgery using sealed- capsule irrigation. J. Cataract Refract. Surg. 29, 1566-1568.

Maloof A.J., Pandey S.K., Neilson G., Milverton E.J., 2005. Selective death of lens epithelial cells using demineralized water and Triton X-100 with PerfectCapsule sealed capsule irrigation: a histological study in rabbit eyes. Arch. Ophthalmol. 123, 1378-1384.

Maltzman B.A., Haupt E., Notis C., 1989. Relationship between age at time of cataract extraction and time interval before capsulotomy for opacification. Ophthalmic Surg. 20, 321-324.

Mamuya F.A., Wang Y., Roop V.H., Scheiblin D.A., Zajac J.C., Duncan M.K., 2014. The roles of alphaV integrins in lens EMT and posterior capsular opacification. J. Cell. Mol. Med. 18, 656-670.

Mansfield K.J., Cerra A., Chamberlain C.G., 2004. FGF-2 counteracts loss of TGFbeta affected cells from rat lens explants: implications for PCO (after cataract). Mol. Vis. 10, 521-532.

Marcantonio J.M., Syam P.P., Liu C.S., Duncan G., 2003. Epithelial transdifferentiation and cataract in the human lens. Exp. Eye Res. 77, 339-346.

Marcantonio J.M., Vrensen G.F., 1999. Cell biology of posterior capsular opacification. Eye (Lond) 13, 484-488.

Martinez G., Wijesinghe M., Turner K., Abud H.E., Taketo M.M., Noda T., Robinson M.L., de Iongh R.U., 2009. Conditional mutations of beta-catenin and APC reveal roles for canonical Wnt signaling in lens differentiation. Invest. Ophthalmol. Vis. Sci. 50, 4794-4806.

McLean S.M., Mathew M.R., Kelly J.B., Murray S.B., Bennett H.G., Webb L.A., Esakowitz L., McLean J.S., 2005. Detection of integrins in human cataract lens epithelial cells and two mammalian lens epithelial cell lines. Br. J. Ophthalmol. 89, 1506-1509.

Meacock W.R., Spalton D.J., Hollick E.J., Boyce J.F., Barman S., Sanguinetti G., 2000a. Double-masked prospective ocular safety study of a lens epithelial cell antibody to prevent posterior capsule opacification. J. Cataract Refract. Surg. 26, 716-721.

Meacock W.R., Spalton D.J., Stanford M.R., 2000b. Role of cytokines in the pathogenesis of posterior capsule opacification. Br. J. Ophthalmol. 84, 332-336.

Melendez R.F., Kumar N., Maswadi S.M., Zaslow K., Glickmank R.D., 2005. Photodynamic actions of indocyanine green and trypan blue on human lens epithelial cells in vitro. Am. J. Ophthalmol. 140, 132-134.

Menapace R., 2008. Posterior capsulorhexis combined with optic buttonholing: an alternative to standard in-the-bag implantation of sharp-edged intraocular lenses? A critical analysis of 1000 consecutive cases. Graefes Arch. Clin. Exp. Ophthalmol. 246, 787-801.

Miyata Y., Oshitari T., Okuyama Y., Shimada A., Takahashi H., Natsugari H., Kosano H., 2013. Polymethoxyflavones as agents that prevent formation of cataract: nobiletin congeners show potent growth inhibitory effects in human lens epithelial cells. Bioorg. Med. Chem. Lett. 23, 183-187.

Nieto M.A., 2011. The ins and outs of the epithelial to mesenchymal transition in health and disease. Annu. Rev. Cell Dev. Biol. 27, 347-376.

Nishi O., 2012. Other factors in PCO prevention. J. Cataract Refract. Surg. 38, 924-5; author reply 925.

Nishi O., Nishi K., Akaishi T., Shirasawa E., 1997. Detection of cell adhesion molecules in lens epithelial cells of human cataracts. Invest. Ophthalmol. Vis. Sci. 38, 579-585.

Nishi O., Nishi K., Mano C., Ichihara M., Honda T., Saitoh I., 1997. Inhibition of migrating lens epithelial cells by blocking the adhesion molecule integrin: a preliminary report. J. Cataract Refract. Surg. 23, 860-865.

Nishi O., Nishi K., Saitoh I., Sakanishi K., 1996. Inhibition of migrating lens epithelial cells by sustained release of ethylenediaminetetraacetic acid. J. Cataract Refract. Surg. 22 Suppl 1, 863-868.

Nishi O., Nishi K., Wickstrom K., 2000. Preventing lens epithelial cell migration using intraocular lenses with sharp rectangular edges. J. Cataract Refract. Surg. 26, 1543-1549.

Nishi O., Nishi K., Yamada Y., Mizumoto Y., 1995. Effect of indomethacin-coated posterior chamber intraocular lenses on postoperative inflammation and posterior capsule opacification. J. Cataract Refract. Surg. 21, 574-578.

Oh J.K., Drumright R., Siegwart D.J., Matyjaszewski K., 2008. The development of microgels/nanogels for drug delivery applications. Progress in Polymer Science 33, 448-477.

Oharazawa H., Ibaraki N., Lin L.R., Reddy V.N., 1999. The effects of extracellular matrix on cell attachment, proliferation and migration in a human lens epithelial cell line. Exp. Eye Res. 69, 603-610.

Olivero D.K., Furcht L.T., 1993. Type IV collagen, laminin, and fibronectin promote the adhesion and migration of rabbit lens epithelial cells in vitro. Invest. Ophthalmol. Vis. Sci. 34, 2825-2834.

Ollerton A., Werner L., Fuller S.R., Kavoussi S.C., McIntyre J.S., Mamalis N., 2012. Evaluation of a new single-piece 4% water content hydrophobic acrylic intraocular lens in the rabbit model. J. Cataract Refract. Surg. 38, 1827-1832.

Pande M., Spalton D.J., Marshall J., 1996a. Continuous curvilinear capsulorhexis and intraocular lens biocompatibility. J. Cataract Refract. Surg. 22, 89-97.

Pande M.V., Spalton D.J., Kerr-Muir M.G., Marshall J., 1996b. Postoperative inflammatory response to phacoemulsification and extracapsular cataract surgery: aqueous flare and cells. J. Cataract Refract. Surg. 22 Suppl 1, 770-774.

Pei C., Xu Y., Jiang J.X., Cui L.J., Li L., Qin L., 2013. Application of sustained delivery microsphere of cyclosporine A for preventing posterior capsular opacification in rabbits. Int. J. Ophthalmol. 6, 1-7.

Peng Q., Apple D.J., Visessook N., Werner L., Pandey S.K., Escobar-Gomez M., Schoderbek R., Guindi A., 2000. Surgical prevention of posterior capsule opacification. Part 2: Enhancement of cortical cleanup by focusing on hydrodissection. J. Cataract Refract. Surg. 26, 188-197.

Prada J., Ngo-Tu T., Baatz H., Hartmann C., Pleyer U., 2000. Detection of tumor necrosis factor alpha and interleukin 1 alpha gene expression in human lens epithelial cells. J. Cataract Refract. Surg. 26, 114-117.

Pritchard C.D., Arner K.M., Langer R.S., Ghosh F.K., 2010. Retinal transplantation using surface modified poly(glycerol-co-sebacic acid) membranes. Biomaterials 31, 7978-7984.

Rabsilber T.M., Limberger I.J., Reuland A.J., Holzer M.P., Auffarth G.U., 2007. Long-term results of sealed capsule irrigation using distilled water to prevent posterior capsule opacification: a prospective clinical randomised trial. Br. J. Ophthalmol. 91, 912-915.

Raj S.M., Vasavada A.R., Kaid J.S., Vasavada V.A., Vasavada V.A., 2009. Post-operative capsular opacification. Nepal J. Ophthalmol. 1, 43-59.

Redenti S., Neeley W.L., Rompani S., Saigal S., Yang J., Klassen H., Langer R., Young M.J., 2009. Engineering retinal progenitor cell and scrollable poly(glycerol-sebacate) composites for expansion and subretinal transplantation. Biomaterials 30, 3405-3414.

Rekas M., Klus A., Kosatka M., 2013. Sealed-capsule irrigation with distilled deionized water to prevent posterior capsule opacification - prospective, randomized clinical trial. Curr. Eye Res. 38, 363-370.

Ronbeck M., Zetterstrom C., Wejde G., Kugelberg M., 2009. Comparison of posterior capsule opacification development with 3 intraocular lens types: five-year prospective study. J. Cataract Refract. Surg. 35, 1935-1940.

Saika S., 2004. TGF-beta signal transduction in corneal wound healing as a therapeutic target. Cornea 23, S25-30.

Saika S., Ikeda K., Yamanaka O., Flanders K.C., Ohnishi Y., Nakajima Y., Muragaki Y., Ooshima A., 2006. Adenoviral gene transfer of BMP-7, Id2, or Id3 suppresses injury-induced epithelial-to-mesenchymal transition of lens epithelium in mice. Am. J. Physiol. Cell. Physiol. 290, C282-9.

Saika S., Kawashima Y., Miyamoto T., Okada Y., Tanaka S., Yamanaka O., Ohnishi Y., Ooshima A., Yamanaka A., 1998. Immunolocalization of hyaluronan and CD44 in quiescent and proliferating human lens epithelial cells. J. Cataract Refract. Surg. 24, 1266-1270.

Saika S., Kono-Saika S., Ohnishi Y., Sato M., Muragaki Y., Ooshima A., Flanders K.C., Yoo J., Anzano M., Liu C.Y., Kao W.W., Roberts A.B., 2004. Smad3 signaling is required for epithelial-mesenchymal transition of lens epithelium after injury. Am.J.Pathol. 164, 651-663.

Saika S., Miyamoto T., Ishida I., Ohnishi Y., Ooshima A., 2002. Lens epithelial cell death after cataract surgery. J. Cataract Refract. Surg. 28, 1452-1456.

Saika S., Miyamoto T., Ishida I., Okada Y., Ohnishi Y., Ooshima A., 2001. Lens epithelial cell regeneration of a capsule-like structure during postoperative healing in rabbits. J. Cataract Refract. Surg. 27, 1076-1078.

Saika S., Miyamoto T., Kawashima Y., Okada Y., Yamanaka O., Ohnishi Y., Ooshima A., 2000. Immunolocalization of TGF-beta1, -beta2, and -beta3, and TGF-beta receptors in human lens capsules with lens implants. Graefes Arch. Clin. Exp. Ophthalmol. 238, 283-293.

Saika S., Miyamoto T., Tanaka S., Tanaka T., Ishida I., Ohnishi Y., Ooshima A., Ishiwata T., Asano G., Chikama T., Shiraishi A., Liu C.Y., Kao C.W., Kao W.W., 2003. Response of lens epithelial cells to injury: role of lumican in epithelial-mesenchymal transition. Invest. Ophthalmol. Vis. Sci. 44, 2094-2102.

Saika S., Okada Y., Miyamoto T., Ohnishi Y., Ooshima A., McAvoy J.W., 2001. Smad translocation and growth suppression in lens epithelial cells by endogenous TGFbeta2 during wound repair. Exp. Eye Res. 72, 679-686.

Shah S.K., Praveen M.R., Kaul A., Vasavada A.R., Shah G.D., Nihalani B.R., 2009. Impact of anterior capsule polishing on anterior capsule opacification after cataract surgery: a randomized clinical trial. Eye (Lond) 23, 1702-1706.

Sharma P., Panwar M., 2013. Trypan blue injection into the capsular bag during phacoemulsification: initial postoperative posterior capsule opacification results. J. Cataract Refract. Surg. 39, 699-704.

Shi Y., Massague J., 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113, 685-700.

Sivak J.M., Fini M.E., 2002. MMPs in the eye: emerging roles for matrix metalloproteinases in ocular physiology. Prog. Retin. Eye Res. 21, 1-14.

Spalton D.J., Russell S.L., Evans-Gowing R., Eldred J.A., Wormstone I.M., 2014. Effect of total lens epithelial cell destruction on intraocular lens fixation in the human capsular bag. J. Cataract Refract. Surg. 40, 306-312.

Steinert R.F., Puliafito C.A., Kumar S.R., Dudak S.D., Patel S., 1991. Cystoid macular edema, retinal detachment, and glaucoma after Nd:YAG laser posterior capsulotomy. Am. J. Ophthalmol. 112, 373-380.

Sternberg K., Terwee T., Stachs O., Guthoff R., Lobler M., Schmitz K.P., 2010. Drug-induced secondary cataract prevention: experimental ex vivo and in vivo results with disulfiram, methotrexate and actinomycin D. Ophthalmic Res. 44, 225-236.

Stifter E., Menapace R., Luksch A., Neumayer T., Vock L., Sacu S., 2007. Objective assessment of intraocular flare after cataract surgery with combined primary posterior capsulorhexis and posterior optic buttonholing in adults. Br. J. Ophthalmol. 91, 1481-1484.

Sun C.B., Teng W.Q., Cui J.T., Huang X.J., Yao K., 2013. The effect of anti-TGF-beta antibody functionalized intraocular lens on lens epithelial cell migration and epithelial-mesenchymal transition. Colloids Surf. B Biointerfaces 113C, 33-42.

Sureshkumar J., Haripriya A., Muthukkaruppan V., Kaufman P.L., Tian B., 2012. Cytoskeletal drugs prevent posterior capsular opacification in human lens capsule in vitro. Graefes Arch. Clin. Exp. Ophthalmol. 250, 507-514.

Tanaka S., Sumioka T., Fujita N., Kitano A., Okada Y., Yamanaka O., Flanders K.C., Miyajima M., Saika S., 2010. Suppression of injury-induced epithelial-mesenchymal transition in a mouse lens epithelium lacking tenascin-C. Mol. Vis. 16, 1194-1205.

Tanaka T., Saika S., Ohnishi Y., Ooshima A., McAvoy J.W., Liu C.Y., Azhar M., Doetschman T., Kao W.W., 2004. Fibroblast growth factor 2: roles of regulation of lens cell proliferation and epithelial-mesenchymal transition in response to injury. Mol. Vis. 10, 462-467.

Tassignon M.J., Dhubhghaill S.N., 2014. Bean-shaped ring segments for capsule stretching and centration of bag-in-the-lens cataract surgery. J. Cataract Refract. Surg. 40, 8-12.

Teo Z.L., McQueen-Miscamble L., Turner K., Martinez G., Madakashira B., Dedhar S., Robinson M.L., de Iongh R.U., 2014. Integrin linked kinase (ILK) is required for lens epithelial cell survival, proliferation and differentiation. Exp. Eye Res. 121, 130-142.

Tholozan F.M., Gribbon C., Li Z., Goldberg M.W., Prescott A.R., McKie N., Quinlan R.A., 2007. FGF-2 release from the lens capsule by MMP-2 maintains lens epithelial cell viability. Mol. Biol. Cell 18, 4222-4231.

Totan Y., Yagci R., Erdurmus M., Bayrak R., Hepsen I.F., 2008. Cyclosporin effectively inhibits posterior capsule opacification after phacoemulsification in rabbits: a preliminary study. Clin. Experiment. Ophthalmol. 36, 62-66.

Trinavarat A., Atchaneeyasakul L., Udompunturak S., 2001. Neodymium:YAG laser damage threshold of foldable intraocular lenses. J. Cataract Refract. Surg. 27, 775-780.

Urakami C., Kurosaka D., Tamada K., Kishimoto S., Tezuka Y., Nishigori H., 2012. Lovastatin alters TGF-beta-induced epithelial-mesenchymal transition in porcine lens epithelial cells. Curr. Eye Res. 37, 479-485.

van Kooten T.G., Koopmans S., Terwee T., Norrby S., Hooymans J.M., Busscher H.J., 2006. Development of an accommodating intra-ocular lens - in vitro prevention of re-growth of pig and rabbit lens capsule epithelial cells. Biomaterials 27, 5554-5560.

van Tenten Y., Schuitmaker H.J., De Groot V., Willekens B., Vrensen G.F., Tassignon M.J., 2002. A preliminary study on the prevention of posterior capsule opacification by photodynamic therapy with bacteriochlorin A in rabbits. Ophthalmic Res. 34, 113-118.

Vasavada A.R., Dholakia S.A., Raj S.M., Singh R., 2006a. Effect of cortical cleaving hydrodissection on posterior capsule opacification in age-related nuclear cataract. J. Cataract Refract. Surg. 32, 1196-1200.

Vasavada A.R., Raj S.M., Johar K., Nanavaty M.A., 2006b. Effect of hydrodissection alone and hydrodissection combined with rotation on lens epithelial cells: surgical approach for the prevention of posterior capsule opacification. J. Cataract Refract. Surg. 32, 145-150.

Walker J., Menko A.S., 2009. Integrins in lens development and disease. Exp. Eye Res. 88, 216-225.

Walker J.L., Wolff I.M., Zhang L., Menko A.S., 2007. Activation of SRC kinases signals induction of posterior capsule opacification. Invest. Ophthalmol. Vis. Sci. 48, 2214-2223.

Walker T.D., 2008. Pharmacological attempts to reduce posterior capsule opacification after cataract surgery - a review. Clin. Experiment. Ophthalmol. 36, 883-890.

Wallentin N., Wickstrom K., Lundberg C., 1998. Effect of cataract surgery on aqueous TGF-beta and lens epithelial cell proliferation. Invest. Ophthalmol. Vis. Sci. 39, 1410-1418.

Wang D., Guo D., Bi H., Wu Q., Tian Q., Du Y., 2013a. Zinc oxide nanoparticles inhibit Ca-ATPase expression in human lens epithelial cells under UVB irradiation. Toxicol. in. Vitro. 27, 2117-2126.

Wang Y., Li W., Zang X., Chen N., Liu T., Tsonis P.A., Huang Y., 2013b. MicroRNA-204-5p regulates epithelial-to-mesenchymal transition during human posterior capsule opacification by targeting SMAD4. Invest. Ophthalmol. Vis. Sci. 54, 323-332.

Wederell E.D., de Iongh R.U., 2006. Extracellular matrix and integrin signaling in lens development and cataract. Semin. Cell Dev. Biol. 17, 759-776.

Weidmann A., Kwittner S., Beck R., Teller J., Jonas L., Nebe J.B., 2008. Prevention of Lens Epithelial Cell Growth In Vitro Using Mibefradil-Containing PLGA Micro Particles. Open Ophthalmol. J. 2, 112-118.

Weissig V., Pettinger T.K., Murdock N., 2014. Nanopharmaceuticals (part 1): products on the market. Int. J. Nanomedicine 9, 4357-4373.

Wejde G., Kugelberg M., Zetterstrom C., 2003. Posterior capsule opacification: comparison of 3 intraocular lenses of different materials and design. J. Cataract Refract. Surg. 29, 1556-1559.

Wertheimer C., Liegl R., Kernt M., Docheva D., Kampik A., Eibl-Lindner K.H., 2014. EGFR-Blockade With Erlotinib Reduces EGF and TGF-beta2 Expression and the Actin-Cytoskeleton Which Influences Different Aspects of Cellular Migration in Lens Epithelial cells. Curr. Eye Res. 39, 1000-1012.

Wertheimer C., Liegl R., Kernt M., Mayer W., Docheva D., Kampik A., Eibl-Lindner K.H., 2013. EGF receptor inhibitor erlotinib as a potential pharmacological prophylaxis for posterior capsule opacification. Graefes Arch. Clin. Exp. Ophthalmol. 251, 1529-1540.

Wormstone I.M., 2002. Posterior capsule opacification: a cell biological perspective. Exp. Eye Res. 74, 337-347.

Wormstone I.M., Tamiya S., Anderson I., Duncan G., 2002. TGF-beta2-induced matrix modification and cell transdifferentiation in the human lens capsular bag. Invest. Ophthalmol. Vis. Sci. 43, 2301-2308.

Wormstone I.M., Tamiya S., Eldred J.A., Lazaridis K., Chantry A., Reddan J.R., Anderson I., Duncan G., 2004. Characterisation of TGF-beta2 signalling and function in a human lens cell line. Exp. Eye Res. 78, 705-714.

Wormstone I.M., Wang L., Liu C.S., 2009. Posterior capsule opacification. Exp. Eye Res. 88, 257-269.

Worthington J.J., Klementowicz J.E., Travis M.A., 2011. TGFbeta: a sleeping giant awoken by integrins. Trends Biochem Sci 36, 47-54.

Wu Y., Deng J., Rychahou P.G., Qiu S., Evers B.M., Zhou B.P., 2009. Stabilization of snail by NF-kappaB is required for inflammation-induced cell migration and invasion. Cancer. Cell. 15, 416-428.

Xie L., Santhoshkumar P., Reneker L.W., Sharma K.K., 2014. Histone Deacetylase Inhibitors Trichostatin A and Vorinostat Inhibit TGFbeta2-Induced Lens Epithelial-to-Mesenchymal Cell Transition. Invest. Ophthalmol. Vis. Sci. 55, 4731-4740 .

Xie L., Sun J., Yao Z., 2003. Heparin drug delivery system for prevention of posterior capsular opacification in rabbit eyes. Graefes Arch. Clin. Exp. Ophthalmol. 241, 309-313.

Xiong W., Cheng B.H., Jia S.B., Tang L.S., 2010. Involvement of the PI3K/Akt signaling pathway in platelet-derived growth factor-induced migration of human lens epithelial cells. Curr. Eye Res. 35, 389-401.

Yang J., Liu T.J., Jiang Y.X., Lu Y., 2012. ATRA enhances the bystander effect of suicide gene therapy driven by the specific promoter LEP 503 in human lens epithelial cells. Mol. Vis. 18, 2053-2066.

Yang Y., Ye Y., Lin X., Wu K., Yu M., 2013. Inhibition of pirfenidone on TGF-beta2 induced proliferation, migration and epithlial-mesenchymal transition of human lens epithelial cells line SRA01/04. PLoS One 8, e56837.

Zajicova A., Pokorna K., Lencova A., Krulova M., Svobodova E., Kubinova S., Sykova E., Pradny M., Michalek J., Svobodova J., Munzarova M., Holan V., 2010. Treatment of ocular surface injuries by limbal and mesenchymal stem cells growing on nanofiber scaffolds. Cell Transplant. 19, 1281-1290.

Zarbin M.A., Montemagno C., Leary J.F., Ritch R., 2010. Nanomedicine in ophthalmology: the new frontier. Am. J. Ophthalmol. 150, 144-162.e2.

Zhang W., Li X., Ye T., Chen F., Sun X., Kong J., Yang X., Pan W., Li S., 2013. Design, characterization, and in vitro cellular inhibition and uptake of optimized genistein-loaded NLC for the prevention of posterior capsular opacification using response surface methodology. Int. J. Pharm. 454, 354-366.

Zhang X.H., Ji J., Zhang H., Tang X., Sun H.M., Yuan J.Q., 2000. Detection of integrins in cataract lens epithelial cells. J. Cataract Refract. Surg. 26, 287-291.

Zhang X.H., Sun H.M., Yuan J.Q., 2001. Extracellular matrix production of lens epithelial cells. J. Cataract Refract. Surg. 27, 1303-1309.

Zheng D., Song T., Zhongliu X., Wu M., Liang J., Liu Y., 2012. Downregulation of transforming growth factor-beta type II receptor prohibit epithelial-to-mesenchymal transition in lens epithelium. Mol. Vis. 18, 1238-1246.

Zhou P., Lu Y., Sun X.H., 2011. Zebularine suppresses TGF-beta-induced lens epithelial cell-myofibroblast transdifferentiation by inhibiting MeCP2. Mol. Vis. 17, 2717-2723.

Zhou P., Lu Y., Sun X.H., 2012. Effects of a novel DNA methyltransferase inhibitor Zebularine on human lens epithelial cells. Mol. Vis. 18, 22-28.

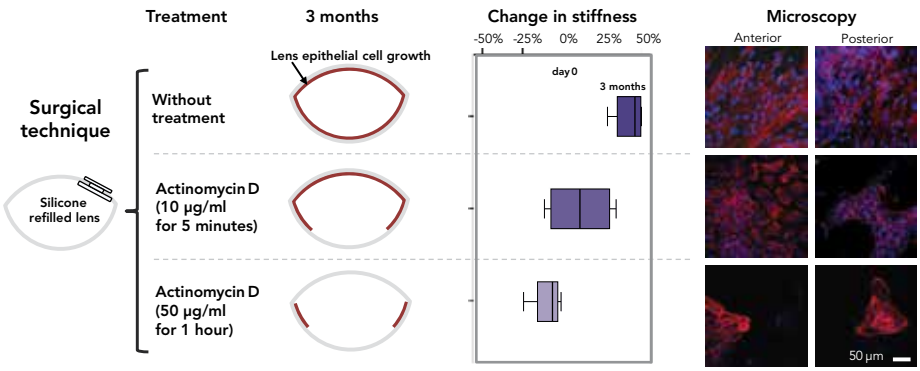
Chapter 3

Changes in lens stiffness due to capsular opacification in accommodative lens refilling

Lisanne M. Nibourg, Prashant K. Sharma, Theo G. van Kooten, and Steven A. Koopmans

This chapter is an edited version of the manuscript: Nibourg L.M., Sharma P.K., van Kooten T.G., Koopmans S.A., 2015. Changes in lens stiffness due to capsular opacification in accommodative lens refilling. Experimental Eye Research 134, 148-154. (Re-use permitted)

Graphical abstract



Abstract

Accommodation may be restored to presbyopic lenses by refilling the lens capsular bag with a soft polymer. After this accommodative lens refilling prevention of capsular opacification is a requirement, since capsular opacification leads to a decreased clarity of the refilled lens. It has been hypothesized that capsular fibrosis causing the capsular opacification results in increased stiffness of the lens capsular bag, therewith contributing to a decrease in accommodative amplitude of the lens. However, the change in viscoelastic properties of refilled lenses due to capsular fibrosis has never been measured directly. In this study we examined natural lenses from enucleated porcine eyes and refilled lenses directly after refilling and after three months of culturing, when capsular fibrosis had developed, and determined their viscoelastic properties with a low load compression tester. Control refilled lenses were included in which capsular opacification was prevented by treatment with actinomycin D. We related lens stiffening to the degree of capsular opacification, as derived from the microscopic images taken with a confocal laser scanning microscope. Overall, the refilled lenses directly after refilling were softer than refilled lenses after three months of culturing, and refilled lenses treated with actinomycin D were softer compared with untreated refilled lenses. The degree of capsular opacification as assessed by microscopy corresponds to an increase in lens stiffness. This indicates that the viscoelastic properties of the refilled lens are influenced by capsular fibrosis and modulated by treatment of the lens epithelium. In conclusion, this study shows that the development of capsular fibrosis negatively affects the viscoelastic properties of isolated, cultured refilled lenses.

3.1 Introduction

In recent years, there has been an increasing interest in the prevention of posterior capsular opacification (PCO), as has been reviewed by Wormstone et al. (2009). PCO is a common side-effect of cataract surgery. With cataract surgery, the opacified lens fibers are extracted, and an intraocular lens (IOL) is placed into the capsular bag. Usually, lens epithelial cells (LECs) situated on the anterior capsule are left behind. These are the cells mainly responsible for the formation of PCO. During the development of PCO, the residual LECs proliferate, transdifferentiate, and migrate to the posterior capsule. PCO results in a decrease of vision in months to years after the initial cataract surgery and is mostly treated with a neodymium-doped yttrium aluminium garnet (Nd:YAG) laser capsulotomy. Complications of this Nd:YAG laser therapy include damage to the IOL, elevation of the intraocular pressure, cystoid macular edema, and increased incidence of retinal detachment (Aslam et al., 2003). Beside the possible complications, the considerable high procurement costs of a Nd:YAG laser make laser capsulotomy not universally available for all patients suffering from PCO. Hence there is a strong need to determine how to prevent PCO instead of performing this treatment which results in additional costs and possible complications.

The ongoing development of new intraocular lenses also results in a need for the prevention of PCO and more in general overall capsular opacification (CO) as the anterior capsule often is saved in new, proposed lens replacement strategies. Prevention is for instance important for the development of an injectable accommodating lens to restore accommodation after the development of presbyopia (Haefliger et al., 1987, Koopmans et al., 2003, Nishi and Nishi, 1998). In order to enable accommodation, the stiffened lens fibers are removed and the lens capsular bag is refilled with a soft transparent polymer. With this technique, the whole lens capsule including the anterior and posterior lens capsule remain in situ, allowing aberrant LEC growth and subsequent opacification on anterior and posterior sides of the capsular bag.

When an injectable accommodating lens is implanted, it is not desirable to destroy the capsular bag by a Nd:YAG laser capsulotomy, because then the injected material will leak out of the capsular bag into the anterior or posterior segments of the eye. Furthermore, CO after injection of a polymer in the capsular bag not only causes a decrease in lens clarity, but may also stiffen the lens due to the presence of the fibrotic tissue. The presence of this tissue has been related to the observed decrease in accommodative amplitude in

monkey eyes that were refilled with a silicone polymer (Koopmans et al., 2006, Koopmans et al., 2014). The decrease in accommodative amplitude in these monkeys was probably the result of CO, although the possibility that other factors such as changes to the ciliary muscle after the implantation procedure or the reduced adhesion between lens capsule and lens refilling material as a cause cannot be completely ruled out (Koopmans, 2006).

In order to investigate the independent role of CO on the viscoelastic properties of lenses, we examined natural and refilled porcine lenses directly after the refilling procedure and after three months of culturing, after which capsular fibrosis had developed. The viscoelastic properties were measured with the low load compression tester (Sharma et al., 2011). After *in vivo* implantation of an accommodative lens, measurements of the changes in lens viscoelastic properties during the development of CO are not possible since the lenses are then situated in an eye, thus allowing indirect measurements only. In a set-up using enucleated porcine lenses in culture we were able to examine the process of lens stiffening due to capsular fibrosis directly. Additionally, we analyzed the viscoelastic properties of refilled lenses treated with the cytotoxic agent actinomycin D, in order to prevent the development of CO, and compared these measurements with untreated refilled lenses.

3.2 Material and methods

Lenses

Eyes from approximately six month old pigs (*Sus domesticus*) were obtained from the local slaughterhouse, lenses were extracted, and the viscoelasticity was measured within eight hours post-mortem. The lenses were divided into five groups. The first group of nine natural lenses served as a control, the lenses were extracted and the viscoelasticity was measured (natural, N). In the following group of seven lenses, the lens fibers were removed, the lenses were refilled with a silicone polymer and the viscoelasticity was measured (refilled, R). The next group consisted of ten lenses which were refilled and cultured for three months before the viscoelasticity was measured (refilled cultured, RC). The last two groups of eight and seven lenses underwent respectively mild (10 µg/ml for 5 minutes) and strong treatment (50 µg/ml for 1 hour) with actinomycin D dissolved in purified water (milli-Q, EMD Millipore Corporation, Billerica, USA) before the lenses were refilled (treatment cultured mild and strong, TC mild and TC strong). Subsequently,

these lenses were cultured for three months and were then measured for viscoelasticity. All groups started with ten samples but due to either fungal contamination or filling errors (under or overfilling) some lenses had to be left out from further statistical analysis.

Surgical refilling procedure

The following surgical procedure is based on the method previously described by Koopmans et al. (2003, 2006). To refill the lens, a 0.8 mm clear cornea paracentesis was made with a single edged diamond knife and an anterior chamber maintainer was inserted. This maintainer was connected to an infusion bottle containing phosphate buffered saline (PBS). Then, a 2.0 mm clear cornea tunnel incision was made using a diamond phaco knife. On approximately 1/4 of the diameter of the lens, a small peripheral puncture of the anterior lens capsule was made by a 25G needle. The capsule opening was torn into a 1.0 to 1.5 mm diameter circular capsulorhexis with a micro-incision capsulorhexis forceps. Subsequently, a 25G blunt cannula connected to a 10 mL syringe with a polyethylene tube was used to extract the lens fibers by manual aspiration. After extraction of the lens fibers, the anterior chamber maintainer was removed and the anterior chamber was filled with sodium hyaluronate (Healon 10 mg/ml; Abbot Medical Optics, Uppsala, Sweden). A 3.0 mm diameter capsular plug (Terwee and Koopmans, 2007) was inserted through the capsulorhexis into the capsular bag, and the capsular bag was filled with a silicone polymer (Abbot Medical Optics, Groningen, The Netherlands). To inject this polymer, a 25G blunt cannula was inserted into the bag. The silicone polymer was injected until it started to leak around the plug and the capsular bag was completely filled. The cannula was removed, and the plug was positioned to close the capsulorhexis.

Treatment with actinomycin D

In the treatment cultured groups, the lens capsules received a mild (10 µg/ml for 5 minutes) or strong (50 µg/ml for 1 hour) treatment with actinomycin D (Sigma-Aldrich Corporation, St. Louis, USA) dissolved in purified water (milli-Q, EMD Millipore Corporation, Billerica, USA). After the removal of the lens fibers and the injection of sodium hyaluronate in the anterior chamber, the capsular bag was filled with the actinomycin D solution. The solution was left in the capsular bag for five minutes (TC mild) or one hour (TC strong). Then the actinomycin D was aspirated and the bag was flushed with PBS. Subsequently, the refilling procedure was continued with the injection of the silicone polymer.

Extraction of the lens from the eye

To enable viscoelasticity measurements, culturing, and microscopy, the natural and refilled lenses were extracted from the eye. First, the cornea was cut off with scissors with sharp curved blades. Then the iris was removed and the zonules were cut using Westcott scissors. The attached vitreous was removed by lifting the lens, and the lens was placed in the culture medium.

Culturing method

The lenses were kept in culture medium consisting of minimal essential medium (MEM) supplemented with 12% fetal bovine serum (FBS), 2 mM GlutaMAX™-I, 1 mM sodium pyruvate, and 500 Units/mL penicillin – 500 µg/mL streptomycin – 1.25 µg/mL amphotericin B (all Life Technologies Ltd, Paisley, UK). The refilled cultured lenses and the treatment cultured lenses were cultured for 3 months in a 37 °C 5% CO_2 incubator. The medium was changed twice a week.

Silicone polymer

The silicone polymer consists of two components which were mixed together by a static mixer and then injected in the lens. After a cross-linking process of 30 minutes at 35°C, the material reaches a stable Young's modulus of 0.8 kPa (Koopmans et al., 2003). The Young's modulus of a 20-year-old human lens is approximately 1 kPa and that of a 60-year-old lens is 5 to 10 kPa (Fisher, 1971, Weeber et al., 2005).

Viscoelasticity testing

The viscoelastic properties of the lenses were measured with a low load compression tester (LLCT) (Sharma et al., 2011). Simplified, the LLCT consists of a bottom plate which measures weight and a top plate which measures its position relative to the bottom plate (Figure 1). The change in position of the top plate and the force on the bottom plate were registered by a computer in LabVIEW (LabVIEW 7.2, National Instruments, Austin, US) for further data analysis.

With the LLCT, first the weight and the thickness of the lens were determined, and then a stress relaxation experiment was performed where the lens was compressed by the top plate within 0.5 seconds to 90 percent of its initial thickness. The top plate was held in this position, and the force on the bottom plate was measured for a period of 100 seconds. Since the lens is viscoelastic in nature, the force decreases with time.

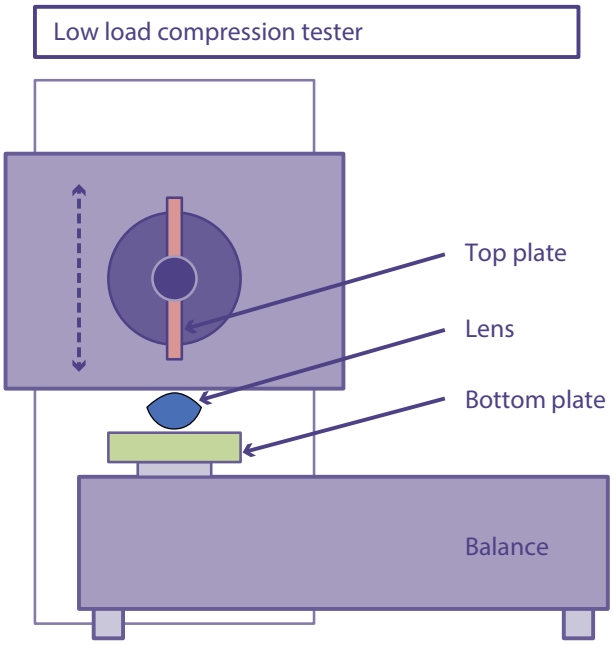


FIGURE 1. *Schematic view of the low load compression tester. The LLCT consists of a bottom plate which measures the weight, and a top plate which measures its position relative to the bottom plate. At the start of the measurement the lens is placed on the bottom plate.*

Figure 2 shows an example of typical data for a refilled lens, giving an impression of the viscoelastic properties of the lens. The precise percentage relaxation and the stiffness can be calculated from the raw data as follows. The percentage relaxation is the percentual difference between the maximal force, just after the start of the measurement, and the force measured after 100 seconds. The stiffness is the slope of the line corresponding with the force at different time points during the 10 percent deformation at the start of the measurement. We used force to calculate percentage relaxation and stiffness because the cross sectional area on which the force is active is unknown, therefore stress cannot be calculated, as previously explained in Sharma et al. (2011).

Imaging of lens epithelial cells

Following the final LLCT measurements, the lenses were fixated with 3.7% paraformadehyde during 1 hour and permeabilized for 15 minutes in 0.5% triton X-100. Subsequently, the LECs were stained with 2 µg/ml TRITC-labeled phalloidin and 4 µg/ml DAPI dissolved in

PBS containing 1% bovine serum albumin. The lenses were stored in PBS until microscopic observation. The Leica TCS SP2 Confocal Laser Scanning Microscope (Leica Microsystems, Rijswijk, the Netherlands) was used for the imaging of the LECs.

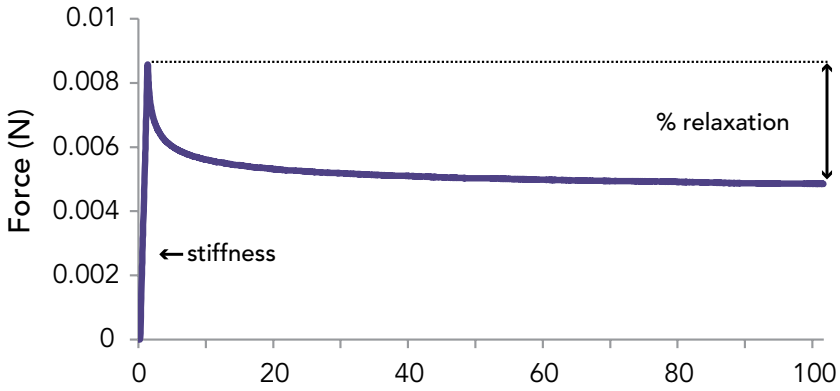


FIGURE 2. *Typical data for a lens tested with the LLCT. The percentage relaxation is the difference between the maximal force and the force after 100 seconds. The stiffness is calculated from the slope of the graph during the 10 percent deformation in 0.5 seconds.*

After microscopic imaging of the LECs, the degree of CO per lens was determined based on the level of LEC migration onto the posterior capsule. The equatorial region always showed LEC growth, thus the degree of CO was taken as 0 when both the anterior and posterior capsule were free of LEC. We graded a 1 if only the anterior side showed LEC and 2 if both sides showed LEC. The degree of CO per group is shown with these numbers together with schematic drawings of lens cross sections which correspond with the LEC growth in refilled lenses with and without actinomycin D treatment (Figures 3 and 4).

Statistical analyses

The Statistical Package for the Social Sciences (IBM SPSS Statistics 20) was used to analyze the data. The data was analyzed in two ways. First we performed a group comparison in which the lenses were divided into five groups: a control group (N), a group with the refilled lenses tested at day 0 (R), the refilled lenses cultured for three months (RC), the refilled lenses with mild actinomycin D treatment and cultured for three months (TC mild),

and the treatment cultured lenses with a strong actinomycin D treatment (TC strong). Kruskal-Wallis analyses with Wilcoxon rank-sum post-hoc tests and Holm-Bonferroni correction were used to test for significant differences between the groups in lens thickness, percentage relaxation, and stiffness.

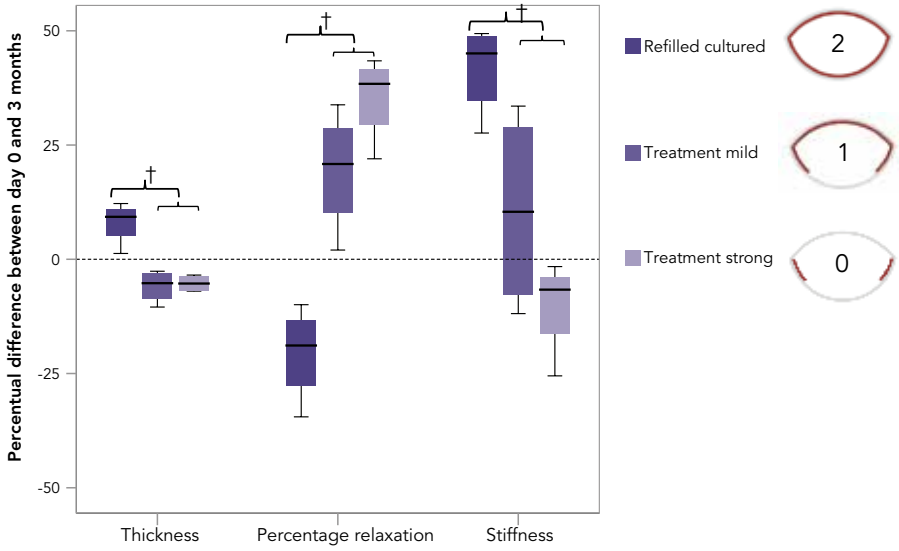


FIGURE 3. *Box plot of percentual difference between the refilled lenses at day 0 and after a culturing period of three months. The dotted line shows the data of the groups at day 0, which was set on 100 percent. The boxes show the percentual difference of the data after three months. Together with the group names a schematic cross section and CO grading is shown. Significant differences between groups are indicated with †.*

As a second analysis we performed a within-lens comparison for the refilled, the treatment mild, and treatment strong lenses. The percentual difference was calculated between the data from day 0 and the data from the same lenses after three months of culturing. The data at day 0 were set at 100 percent, so for each group of lenses three variables for within-lens differences were obtained. The differences in thickness, percentage relaxation, and stiffness between groups were compared with Kruskal-Wallis analyses with Wilcoxon rank-sum post-hoc tests and Holm-Bonferroni correction. P values ≤ 0.05 were considered statistically significant (indicated with †) and with Holm-Bonferroni correction, significance levels are corrected by the number of performed comparisons.

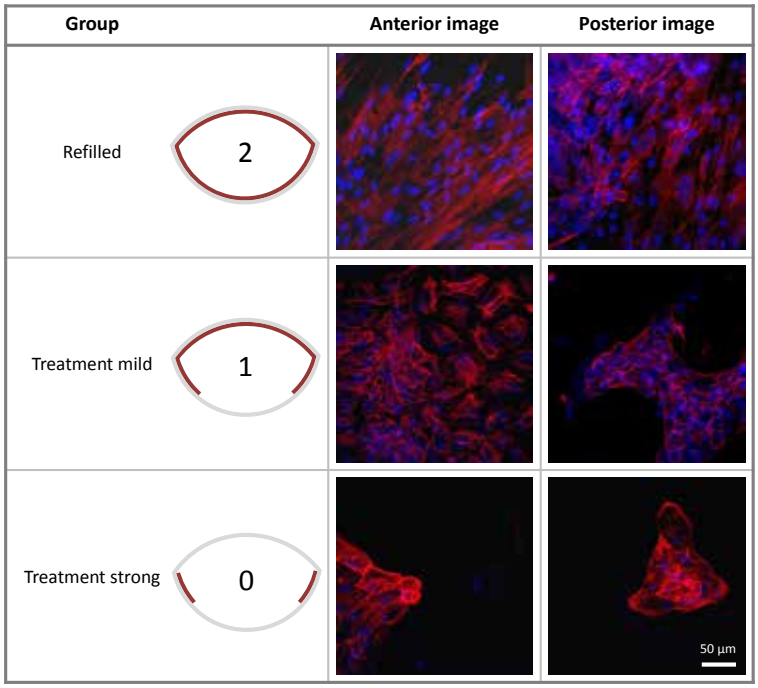


FIGURE 4. *Confocal Laser Scanning Microscope images from lenses after 3 months of culturing. The figure shows images from the anterior and posterior side of a lens from the refilled cultured group and the treatment cultured groups. Together with the group names a schematic cross section and CO grading is shown.*

3.3 Results and discussion

Group comparison

Fifty fresh natural porcine eyes were obtained from the local slaughterhouse and due to dropout we were left with 41 lenses, which were divided into the five groups: natural (N, n = 9), refilled (R, n = 7), refilled cultured (RC, n = 10), treatment cultured mild (TC mild, n = 8), and treatment cultured strong (TC strong, n = 7). Figure 5 shows the differences between the groups in lens thickness, percentage relaxation, and stiffness. Kruskal-Wallis analyses revealed significant differences between the groups for all three variables (P values ≤ 0.009). For the thickness measurements, the natural lenses had the smallest range. The larger range and differences in thickness between the refilled lenses were probably due to differences in injected volumes of the silicone polymer as this is difficult

to control. Post-hoc tests showed a significant difference in thickness between the R and RC groups (P = 0.002). Differences in injected volumes could have some influence on the percentage relaxation. With less volume injected, we expect a higher percentage relaxation because then the flexibility of lens capsule contributes more to the total viscoelasticity of the lens. However, this was not found in the individual lenses. In section 3.3 (page 87) the results from the within-lens measurements of the thickness of these lenses are shown and thickness results are further discussed.

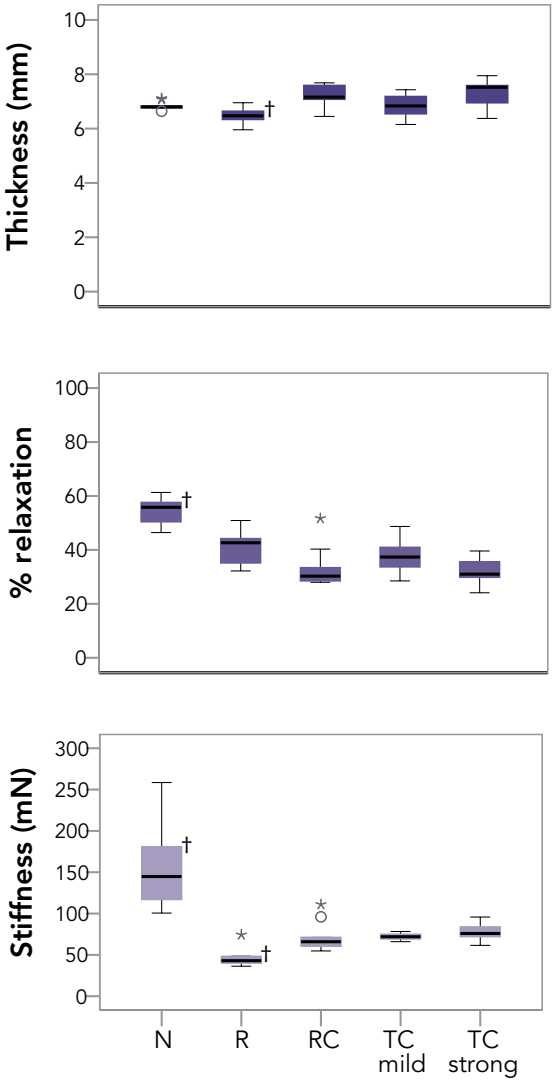


FIGURE 5. *Box plot of the differences between groups in lens thickness, percentage relaxation, and stiffness. Group medians, quartiles and outliers (o = outlier, * = far outlier) are shown for each outcome variable. Significant differences between groups are indicated with †. N: natural lenses, R: refilled lenses, RC: refilled cultured, TC mild: treatment cultured mild, TC strong: treatment cultured strong.*

The graph of the percentage relaxation (Figure 5) shows that natural porcine lenses have the largest ability to relax after a 10 percent deformation, significantly different from all the other groups (P values ≤ 0.001). It is also notable that there is a trend for differences between the refilled lenses at day 0 (R) and the lenses after culturing (RC), however, this was not significant. Based on our hypothesis we expected that the RC group had less relaxation due to capsular fibrosis.

With respect to stiffness, the N and R groups were significantly different from all the other groups (P values ≤ 0.009). It is remarkable that the range of natural lens stiffness is much larger than that of the refilled groups, indicating that the natural variety of lens stiffness is rather large despite the fact that the pigs from which the eyes were obtained all had a similar age at sacrifice (six months). Furthermore, it is notable that the natural porcine lenses appear to be much stiffer compared to the refilled lenses, also after three months of culturing. This shows that the silicone polymer is much more flexible than the lenscontent of a young pig. Even after the development of capsular fibrosis the refilled lenses are less stiff compared with natural porcine lenses. The stiffness of the natural porcine lens is reflected in the fact that pigs do not show accommodation.

Within-lens comparison

The within-lens differences between day 0 and 3 months for the refilled cultured (n = 4), treatment mild (n = 4), and treatment strong (n = 4) groups are highlighted in Figure 3. These groups were more at risk for fungal contamination of the lenses in culture due to the various manipulations necessary for the LLCT measurements, resulting in a higher dropout of lenses. Despite the fact that the groups were small, differences between groups were found. Kruskal-Wallis analyses revealed significant differences between the groups for all three variables: thickness (P = 0.025), percentage relaxation (P = 0.012), and stiffness (P = 0.026).

Figure 3 shows that the refilled lenses increased in thickness over the 3 months of culturing, while a decrease in thickness was seen when CO was prevented with actinomycin D treatment (P = 0.021). However, because of correction for multiple testing caution is needed with interpreting these values (Holm-Bonferroni corrected $\alpha = 0.05 / 3 = 0.017$). Unfortunately, this problem is attributable to the small sample size in groups, making the minimal reachable P value P = 0.021. So with these tests we only can interpret overall group P values correctly. Since we used the percentual difference between day 0 and 3 months and there was no overlap in values between groups, these findings suggest a

possible effect of treatment on lens thickness. To underline this argument we additionally performed a Wilcoxon rank-sum test for the difference between the refilled group and both treatment groups together and this was found to be significant (P = 0.007). The increase in lens thickness in the refilled group can be due to CO inside the lens capsule. In the lenses treated with actinomycin D, the development of CO was much less, hence no increase in lens thickness was observed.

The group differences for the percentage relaxation were also found to be significant (P = 0.012). For all the refilled lenses the percentage relaxation decreased over 3 months, most likely due to lens stiffening as a result of capsular fibrosis. Interestingly, we found an increase in percentage relaxation in all the treated lenses. The box plot shows even a higher increase in the lenses with the strong treatment. In the post-hoc tests these differences between the refilled and treatment groups were also found to have the lowest possible P value (P = 0.021). When both treatment groups were combined and compared with the refilled group we found a significant difference for percentage relaxation (P = 0.007). So the data show an effect of actinomycin D treatment on percentage relaxation when both treatment groups are taken together and display a potential difference between mild and strong treatment.

For the stiffness data we can see an inverse distribution of the boxes compared to the percentage relaxation data. The refilled lenses increased in stiffness due to capsular fibrosis. In the mild treatment lenses the stiffness increased or decreased a bit, and for the strong treatment the stiffness decreased over 3 months. The differences between both treatment groups can be attributed to the formation of CO (see Figure 4), as the lenses with the mild treatment still had LECs on the anterior capsule and some LECs posterior, and the strong treatment lenses only had LECs in the equator. The difference between the refilled and the treatment strong lenses was found to be the lowest possible P (P = 0.021), so this would suggest a possible influence of therapy on stiffness measured with the LLCT. We also performed a comparison for stiffness in which we combined both treatment groups against the refilled group, and this was found to be significant as well (P = 0.011). The observed differences are supported by the results of the confocal imaging which are discussed on the next page.

Microscopic imaging

Figure 4 shows representative images for lenses from the RC, TC mild, and TC strong groups. The figure shows stretched cells on the anterior side of the refilled lens, indicating a transdifferentiation of LECs. Furthermore, the image of the posterior side of the refilled lens is fully filled with migrated LECs. So the lenses in the refilled group all had extensive development of CO. In the TC mild lenses the anterior capsule was covered with LECs, but now with a more normal conformation; hexagonally shaped LECs similar to LECs in a natural lens. In the image of the posterior side of this lens some LECs are visible in the periphery, so there was some cell migration. The TC strong lenses only showed some LECs in the equatorial regions of the posterior and anterior capsule, but none in the central axis of the anterior or posterior capsule. All lenses showed LEC migration on both the posterior and the anterior capsule. This is probably due to the fact that we did not use anterior capsule polishing techniques during the refilling procedure, so there were always LEC left on the anterior capsule in varying amounts.

Combining mechanical data and microscopic imaging

To our knowledge, this is the first paper combining microscopic imaging of CO with mechanical properties of the same lenses. The CO as seen in the images in Figure 4 is well related to the outcome of the LLCT measurements represented in Figure 3. These figures show that the treatment with actinomycin D decreased capsular fibrosis and that is reflected in a reduced stiffness of the lens. Actinomycin D treatment has already been used before in studies aimed at the prevention of CO (Koopmans et al., 2011, Koopmans et al., 2014, Sternberg et al., 2010, van Kooten et al., 2006). It was shown that actinomycin D can be effective in preventing CO, however, once it leaks out of the lens capsule in the anterior chamber severe damage to the corneal endothelium may happen resulting in corneal opacity. In this study we used a much higher dose of actinomycin D and a much longer application time compared to previous studies in an effort to eradicate all LECs from the capsular bag. Despite the high dose and application time, LECs could still be detected in the equatorial region of the cultured lens capsules after 3 months. In a previous study using LLCT measurements it was already shown that differences in viscoelastic properties of natural lenses could be detected using this technique (Sharma et al., 2011). The current study confirms the fact that this technique is sensitive enough to measure differences in lens viscoelastic properties. A clear relation with the occurrence of capsular fibrosis has been shown in this study, although a causal relationship cannot be proven.

Comparison to other methods for measuring mechanical lens properties

Several other methods have been described to monitor mechanical properties or accommodation in lenses. First, a considerable number of studies have been published on the use of a lens stretcher (Ehrmann et al., 2008, Fisher, 1977, Glasser and Campbell, 1998, Kammel et al., 2012, Koopmans et al., 2003, Manns et al., 2007, Pierscionek, 1995, Reilly et al., 2008). With this method the lens, together with the ciliary body, is placed into a device which can stretch the lens. During the stretching, different parameters can be monitored, like optical changes, geometrical properties, and stretching force. This method has also been carried out on refilled accommodating lenses. Reilly et al. (2009) used a robotic lens stretcher to make a comparison between natural and refilled porcine lenses, and Koopmans et al. (2003) used a lens stretcher to evaluate accommodation in refilled human donor lenses. Another method to monitor lens properties is a bubble-based acoustic radiation force (Erpelding et al., 2007, Yoon et al., 2013). To measure the viscoelasticity, bubbles are induced by a laser in specific parts of the lens. Then an acoustic radiation force is applied on the bubbles and the displacement is measured. The technique has only been described for the use in natural lenses, and it is not possible to combine this technique with the measurement of optical properties. Geometrical properties of accommodation *in vivo* could be monitored by magnetic resonance imaging (MRI) (Strenk et al., 1999), scheimpflug imaging (Hermans et al., 2009), or optical coherence tomography (Shao et al., 2013, Zhong et al., 2014). Stachs et al. (2011) studied the use of high-resolution MRI for the monitoring of accommodation in normal and refilled leporine lenses. Their results showed that high-resolution MRI is a useful imaging method for the analysis of leporine lens refilling *in vivo*. In the current study, the viscoelasticity was measured with a low load compression tester. The LLCT can measure the properties of the lens material itself or of the lens together with the capsule. The use of an LLCT was first described by Sharma et al. (2011). They used the LLCT to measure the viscoelastic properties of human, simian, leporine, and porcine lenses. A major advantage of the LLCT is that mechanical lens measurements can be combined with lens culture over time. This is not possible with the other described methods. To conclude, we state that the LLCT used in this study provides additional value in measuring mechanical properties in injectable accommodating lenses.

3.4 Conclusion

In this study we show that the development of capsular opacification in isolated refilled lenses results in an increase in lens stiffness. Treatment with actinomycin D resulted in a reduction of capsular fibrosis and stiffness of the lenses. This implies that capsular fibrosis contributes to the decrease in accommodative amplitude after lens refilling. The applied method using the LLCT is sensitive enough to measure differences in viscoelastic lens properties *ex vivo*, and therefore can be used to analyze changes in viscoelastic lens properties due to actions such as lens refilling and treatment of the lens.

Acknowledgements

The authors would like to thank Roel Kuijer for the helpful comments made during the set up of the experiments and Abbott Medical Optics Groningen for providing the silicone polymer. This study was supported by the European Fund for Regional Development and the Samenwerkingsverband Noord-Nederland.

References

Aslam T.M., Devlin H., Dhillon B., 2003. Use of Nd:YAG laser capsulotomy. Surv. Ophthalmol. 48, 594-612.

Ehrmann K., Ho A., Parel J.M., 2008. Biomechanical analysis of the accommodative apparatus in primates. Clin. Exp. Optom. 91, 302-312.

Erpelding T.N., Hollman K.W., O'Donnell M., 2007. Mapping age-related elasticity changes in porcine lenses using bubble-based acoustic radiation force. Exp. Eye Res. 84, 332-341.

Fisher R.F., 1971. The elastic constants of the human lens. J. Physiol. 212, 147-180.

Fisher R.F., 1977. The force of contraction of the human ciliary muscle during accommodation. J. Physiol. 270, 51-74.

Glasser A., Campbell M.C., 1998. Presbyopia and the optical changes in the human crystalline lens with age. Vision Res. 38, 209-229.

Haefliger E., Parel J.M., Fantes F., Norton E.W., Anderson D.R., Forster R.K., Hernandez E., Feuer W.J., 1987. Accommodation of an endocapsular silicone lens (Phaco-Ersatz) in the nonhuman primate. Ophthalmology 94, 471-477.

Hermans E.A., Pouwels P.J., Dubbelman M., Kuijer J.P., van der Heijde R.G., Heethaar R.M., 2009. Constant volume of the human lens and decrease in surface area of the capsular bag during accommodation: an MRI and Scheimpflug study. Invest. Ophthalmol. Vis. Sci. 50, 281-289.

Kammel R., Ackermann R., Mai T., Damm C., Nolte S., 2012. Pig lenses in a lens stretcher: implications for presbyopia treatment. Optom. Vis. Sci. 89, 908-915.

Koopmans S.A., Terwee T., Barkhof J., Haitjema H.J., Kooijman A.C., 2003. Polymer refilling of presbyopic human lenses in vitro restores the ability to undergo accommodative changes. Invest. Ophthalmol. Vis. Sci. 44, 250-257.

Koopmans S.A., Terwee T., Glasser A., Wendt M., Vilupuru A.S., van Kooten T.G., Norrby S., Haitjema H.J., Kooijman A.C., 2006. Accommodative lens refilling in rhesus monkeys. Invest. Ophthalmol. Vis. Sci. 47, 2976-2984.

Koopmans S.A., Terwee T., Hanssen A., Martin H., Langner S., Stachs O., van Kooten T.G., 2014. Prevention of capsule opacification after accommodating lens refilling: pilot study of strategies evaluated in a monkey model. J. Cataract Refract. Surg. 40, 1521-1535.

Koopmans S.A., Terwee T., van Kooten T.G., 2011. Prevention of capsular opacification after accommodative lens refilling surgery in rabbits. Biomaterials 32, 5743-5755.

Manns F., Parel J.M., Denham D., Billotte C., Ziebarth N., Borja D., Fernandez V., Aly M., Arrieta E., Ho A., Holden B., 2007. Optomechanical response of human and monkey lenses in a lens stretcher. Invest. Ophthalmol. Vis. Sci. 48, 3260-3268.

Nishi O., Nishi K., 1998. Accommodation amplitude after lens refilling with injectable silicone by sealing the capsule with a plug in primates. Arch. Ophthalmol. 116, 1358-1361.

Pierscionek B.K., 1995. Age-related response of human lenses to stretching forces. Exp. Eye Res. 60, 325-332.

Reilly M.A., Hamilton P.D., Perry G., Ravi N., 2009. Comparison of the behavior of natural and refilled porcine lenses in a robotic lens stretcher. Exp. Eye Res. 88, 483-494.

Reilly M.A., Hamilton P.D., Ravi N., 2008. Dynamic multi-arm radial lens stretcher: a robotic analog of the ciliary body. Exp. Eye Res. 86, 157-164.

Shao Y., Tao A., Jiang H., Shen M., Zhong J., Lu F., Wang J., 2013. Simultaneous real-time imaging of the ocular anterior segment including the ciliary muscle during accommodation. Biomed. Opt. Express 4, 466-480.

Sharma P.K., Busscher H.J., Terwee T., Koopmans S.A., van Kooten T.G., 2011. A comparative study on the viscoelastic properties of human and animal lenses. Exp. Eye Res. 93, 681-688.

Stachs O., Langner S., Terwee T., Sternberg K., Martin H., Schmitz K.P., Hosten N., Guthoff R., 2011. In vivo 7.1 T magnetic resonance imaging to assess the lens geometry in rabbit eyes 3 years after lens-refilling surgery. J. Cataract Refract. Surg. 37, 749-757.

Sternberg K., Terwee T., Stachs O., Guthoff R., Lobler M., Schmitz K.P., 2010. Drug-induced secondary cataract prevention: experimental ex vivo and in vivo results with disulfiram, methotrexate and actinomycin D. Ophthalmic Res. 44, 225-236.

Strenk S.A., Semmlow J.L., Strenk L.M., Munoz P., Gronlund-Jacob J., DeMarco J.K., 1999. Age-related changes in human ciliary muscle and lens: a magnetic resonance imaging study. Invest. Ophthalmol. Vis. Sci. 40, 1162-1169.

Terwee T, Koopmans SA. 2007. Device for use in eye surgery. US patent no. 7182780.

van Kooten T.G., Koopmans S., Terwee T., Norrby S., Hooymans J.M., Busscher H.J., 2006. Development of an accommodating intra-ocular lens--in vitro prevention of re-growth of pig and rabbit lens capsule epithelial cells. Biomaterials 27, 5554-5560.

Weeber H.A., Eckert G., Soergel F., Meyer C.H., Pechhold W., van der Heijde R.G., 2005. Dynamic mechanical properties of human lenses. Exp. Eye Res. 80, 425-434.

Wormstone I.M., Wang L., Liu C.S., 2009. Posterior capsule opacification. Exp. Eye Res. 88, 257-269.

Yoon S., Aglyamov S., Karpiouk A., Emelianov S., 2013. The mechanical properties of ex vivo bovine and porcine crystalline lenses: age-related changes and location-dependent variations. Ultrasound Med. Biol. 39, 1120-1127.

Zhong J., Tao A., Xu Z., Jiang H., Shao Y., Zhang H., Liu C., Wang J., 2014. Whole eye axial biometry during accommodation using ultra-long scan depth optical coherence tomography. Am. J. Ophthalmol. 157, 1064-1069.

Chapter 4

Effects of silicone polymer hydrophilicity on capsular opacification after accommodative lens refilling in a porcine eye model

Lisanne M. Nibourg, Barbara Dittrich, Oliver Stachs, Rudolf F. Guthoff, Theo G. van Kooten, Barbara Dittrich, Thom Terwee, and Steven A. Koopmans

Abstract

This study explores the effect of silicone polymer properties on the development of capsular opacification (CO) after accommodative lens refilling. An *ex vivo* porcine lens model was used, and lenses were refilled with silicone polymers from the two ends of the wettability spectrum (hydrophobic and hydrophilic silicone) in order to investigate the effect on the remaining lens epithelial cells (LECs). The LEC response was also examined in combination with actinomycin D therapy and in HLE-B3 cell cultures. Viscoelasticity measurements were performed in order to determine the stiffness of the refilled lenses. The results showed that there were no relevant differences in CO formation and lens stiffness between hydrophilic or hydrophobic silicone polymers. Thus, material wettability does not seem to be important for silicone polymers in accommodative lens refilling surgery.

4.1 Introduction

Capsular opacification (CO) is a common complication of current cataract surgery and entails the process of proliferation, migration, and transdifferentiation of the residual lens epithelial cells (LECs) in the remaining lens capsular bag (Marcantonio et al., 2003, Nibourg et al., 2015b). The general treatment of capsular opacification is a Nd:YAG capsulotomy of the posterior capsule, which provides a clear visual axis for the patient (Aslam et al., 2003). Complications of Nd:YAG capsulotomies are described (Ge et al., 2000, Steinert et al., 1991, Trinavarat et al., 2001), and in accommodation restoring techniques for cataract surgery, where a functional capsular bag is essential, performing a capsulotomy is not possible (Nibourg et al., 2015a, van Kooten et al., 2006). Therefore, in recent years there is an increased interest in CO prevention.

Accommodative lens refilling is one of the techniques in development for future cataract surgery (Haefliger et al., 1987, Kessler, 1964, Koopmans et al., 2006, Nibourg et al., 2015a, Nishi et al., 2014). This technique involves replacement of the opacified natural lens fibers by an optically clear injectable polymer, which enables accommodation of the new lens. The polymer is injected into the capsular bag and the bag is closed by a capsular plug. Essential for this technique is that the capsular bag must be intact in order to keep the polymer in the capsular bag and to provide the lens changes necessary for

accommodation. Beside a decrease in visual acuity, CO formation causes an increase of the stiffness of the lens capsular bag which will lead to a diminished accommodative amplitude in those lenses (Nibourg et al., 2015a, Nishi et al., 2009, Weeber and van der Heijde, 2007).

Previous studies have reported beneficial effects of lens refilling with hyaluronan (sodium hyaluronate) on CO prevention compared with the targeted silicone polymer lens refilling material named ACL (Koopmans et al., 2014). This CO prevention may be caused by physical and/or biochemical properties of the hyaluronan (Lapčík et al., 1998). A striking difference between the physical characteristics of ACL and hyaluronan is their highly contrasting wettability (Koopmans et al., 2003, Lapčík et al., 1998). ACL has a hydrophobic nature and hyaluronan is hydrophilic. In this study we wanted to test the assumption that the difference in wettability is the main cause for the difference in CO formation. Because hyaluronan is not suitable as a permanent lens replacing material (Arshinoff et al., 2002, Berson et al., 1983), we developed an injectable hydrophilic silicone polymer in order to compare this with the hydrophobic silicone ACL material. Silicone polymers have the potential to meet all characteristics necessary for accommodation restoring injectable lenses (Hao et al., 2010, Koopmans et al., 2003). We investigated the effects on LECs of the two silicone polymers, with properties from two ends of the wettability spectrum, in an *ex vivo* porcine lens model and in an *in vitro* human LEC culture. Also the effects of pre-treatment of the LECs with the cytostatic actinomycin D were evaluated. This was done because it was reported that lens refilling with hyaluronan, after a short actinomycin D treatment, resulted in a clear lens in a rhesus monkey for 9 months while lens refilling with ACL, after the same pre-treatment was not effective for CO prevention in rhesus monkeys (Koopmans et al., 2014).

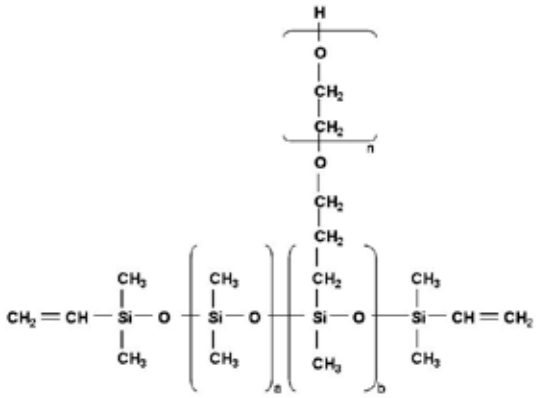
4.2 Material and Methods

Silicone polymers

The structures of the hydrophilic silicone (PEG-modified PDMS, DWI - Leibniz Institute for Interactive Materials, Aachen, Germany) and the hydrophobic silicone (ACL = silicone ter-polymer, Abbott Medical Optics Inc., Groningen, the Netherlands) are shown in Figure 1. The contact angle of a water droplet on the hydrophilic silicone polymer is 38 degrees and on the hydrophobic silicone polymer is 110 degrees. The contact angles of both

polymers were determined after placing the silicone gel on a glass slide for 24 hours at 24°C for polymerization.

1.



2.

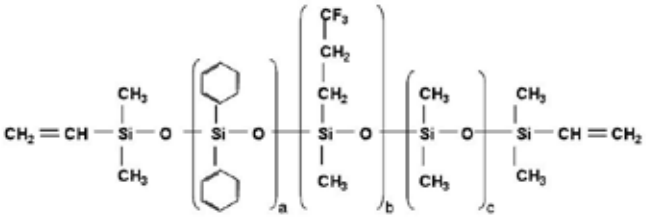


FIGURE 1. *Schematic overview of the structure of the (1) hydrophilic silicone (PEG-modified PDMS) and the (2) hydrophobic silicone (ACL = silicone ter-polymer).*

Porcine eye model

Eyes from approximately six month old pigs (Sus domesticus) were obtained from the local slaughterhouse, and lenses were refilled with the different silicone polymers (Table 1).

Surgical refilling procedure and extraction of the lens

The following surgical procedure is based on previously described methods (Koopmans et al., 2003, Koopmans et al., 2006, Nibourg et al., 2015a). A cornea paracentesis was made to insert an anterior chamber (AC) maintainer connected to an infuse containing phosphate buffered saline (PBS). Then, a 2.0 mm clear cornea tunnel incision and a 1.0 to 1.5 mm

diameter circular capsulorhexis were created. The lens contents were manually extracted using a 25G blunt cannula connected to a 10 mL syringe with a polyethylene tube. The AC was filled with sodium hyaluronate (Healon 10 mg/ml; Abbot Medical Optics, Uppsala, Sweden), and a 3.0 mm diameter capsular plug was inserted through the capsulorhexis into the capsular bag. The capsular bag was then filled with one of the silicone polymers using a 25G blunt cannula. The silicone was injected until it started to leak around the plug and the capsular bag was completely filled. Then the cannula was removed, and the plug was positioned to close the capsulorhexis. Subsequently, the refilled lenses were extracted from the eye by cutting off the cornea, removal of the iris, and cutting the zonules. The attached vitreous was removed and the lens was placed in culture medium.

TABLE 1. *Overview of used materials and results from the tested silicone polymers.*

	Composition	Hydrophilicity	N	Therapy	Results	
					Lens culture	HLE-B3 culture
1	ACL	Hydrophobic	4	-	LEC proliferation, migration and transformation; multiple layers LECs	Sphere shaped aggregates
2	PEG-modified PDMS	Hydrophilic	8	-	LEC proliferation, migration and transformation; single LEC layer	Sphere shaped aggregates
3	ACL	Hydrophobic	4	Actinomycin D, 10 µg/mL for 1 hour	No LEC migration, minimal proliferation andtransformation	-
4	PEG-modified PDMS	Hydrophilic	6	Actinomycin D, 10 µg/mL for 1 hour	No LEC migration, minimal proliferation andtransformation	-

HLE-B3 = human lens epithelial cell line B3

Actinomycin D treatment

For both silicones, a group of in total 10 lenses received a treatment with 10 µg/ml actinomycin D (Sigma-Aldrich Corporation, St. Louis, USA) dissolved in purified water (milli-Q, EMD Millipore Corporation, Billerica, USA). After the removal of the lens fibers and the injection of sodium hyaluronate in the anterior chamber, the capsular bag was filled with the actinomycin D solution. The solution was left in the capsular bag for one hour. Then the actinomycin D was aspirated and the bag was flushed with PBS. Subsequently, the refilling procedure was continued with the injection of the silicone polymer.

Porcine lens culture

The lenses were kept in culture medium consisting of minimal essential medium (MEM) supplemented with 12% fetal bovine serum (FBS), 1% GlutaMAX (GlutaMAX™-I, Life Technologies Ltd, Paisley, UK), 1% sodium pyruvate, and 5% amphotericin-B/penicillin/streptomycin. They were cultured for 21 days in a 37 °C 5% CO2 incubator. The medium was changed twice a week.

Imaging

The lenses were fixated with 3.7% paraformadehyde after three weeks of culturing. LECs were stained for F-actin with 2 µg/ml TRITC-labeled phalloidin and nuclei with 4 µg/ml DAPI dissolved in PBS containing 1% bovine serum albumin (PBSA). Lenses were stored in PBS until microscopic observation.

Viscoelasticity measurements

The viscoelastic properties of the lenses were measured with a low load compression tester (LLCT) directly after the refilling procedure and after three weeks of culture. This method has previously been explained (Nibourg et al., 2015a, Sharma et al., 2011). The LLCT entails a bottom plate measuring weight and a top plate measuring its relative position to the bottom plate. The change in position of the top plate and the force on the bottom plate were registered by a computer in LabVIEW (LabVIEW 7.2, National Instruments, Austin, US) for data analysis.

The LLCT first measures the weight and the thickness of the lens, followed by a stress relaxation experiment. For this measurement, the lens was compressed by the top plate within 0.5 seconds to 90 percent of its initial thickness. The top plate was held in this position, and the force on the bottom plate was measured for a period of 100 seconds. Since the lens is viscoelastic in nature, the force decreases with time. The percentage relaxation and the stiffness can be calculated from the raw data as follows. The percentage relaxation is the percentual difference between the maximal force, just after the start of the measurement, and the force measured after 100 seconds. The stiffness is the slope of the line corresponding with the force at different time points during the 10 percent deformation at the start of the measurement.

The lenses for viscoelasticity testing were divided into six groups (Figure 2). The lenses refilled with hydrophobic silicone were measured directly after refilling (ACL-0) and after 21 days of culture (ACL-21). One group of ACL refilled lenses underwent treatment

with actinomycin D and were measured for viscoelasticity after 21 days of culture (ACL-AD-21). The lenses refilled with hydrophilic PEG-modified PDMS were also divided into three groups with viscoelasticity measurements direct after extraction (PEG-PDMS-0), after 21 days culture (PEG-PDMS-21), and lenses with actinomycin D treatment and viscoelasticity measurements after 21 days of culture (PEG-PDMS-AD-21). For analyzing the data we used the Statistical Package for the Social Sciences (IBM SPSS Statistics 20). Differences between groups in lens thickness, percentage relaxation, and stiffness were examined using analyses of variance (ANOVA) with Games-Howell post-hoc analyses and student T-tests. P values ≤ 0.05 were considered statistically significant.

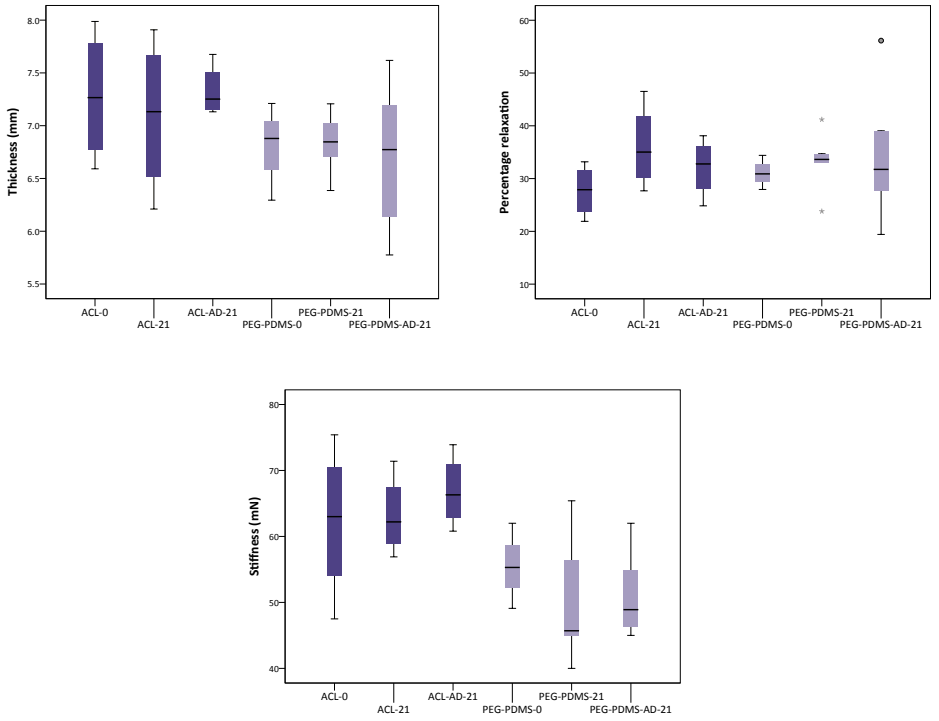


FIGURE 2. *Box plots of the differences between groups in lens thickness, percentage relaxation, and stiffness. Group medians, quartiles and outliers (o = outlier, * = far outlier) are shown for each outcome variable. ACL-0: ACL refilled lenses day 0; PEG-PDMS-0: PEG-modified PDMS refilled lenses day 0; ACL-21: ACL lenses after 21 days culture; PEG-PDMS-21: PEG-modified PDMS lenses 21 days culture; ACL-AD-21: ACL lenses with actinomycin D treatment after 21 days culture; PEG-PDMS-AD-21; PEG-modified PDMS refilled lenses with actinomycin D treatment after 21 days culture.*

Human LEC culture model

Cell culture experiments were performed with a human lens epithelial cell line (HLE-B3).

Preparation of wells plates

Residual silicone polymer of both silicones was put on the bottom of wells from 5 24-wells plates. In each well approximately 32000 HLE-B3 cells were plated. The cells on the hydrophilic silicone were cultured for respectively 2, 4, 7, and 13 days with the same culture medium and procedure as described for the lens cultures. The cells on the hydrophobic silicone were cultured for 5 days.

Imaging

The HLE-B3 cells were fixated with 3.7% paraformadehyde after 5 days of culturing for the hydrophobic silicone and 13 days of culturing for the hydrophilic silicone. Nuclei were stained with 1 µg/ml ethidium bromide dissolved in PBSA. F-actin was stained with 2 µg/ml FITC-labeled phalloidin and fibronectin, a CO marker (Wormstone and Eldred, 2015), with polyclonal rabbit-anti-human fibronectin antibodies (Sigma, 1:400), followed by Cy5-labelled donkey-anti-rabbit secondary antibodies (Jackson; 1:100). Imaging of the LECs was performed with the Leica TCS SP2 Confocal Laser Scanning Microscope (Leica Microsystems, Wetzlar, Germany).

4.3 Results

Porcine lens model

After three weeks of culture, the lenses refilled with hydrophobic silicone all showed LECs on the confocal images (Figure 3). Multiple layers of transdifferentiated LECs on the anterior and posterior capsules can be seen. Treatment with actinomycin D before refilling did lead to a reduction of LEC growth, but there was no full CO prevention in the lenses refilled with hydrophobic silicone. In the lenses refilled with hydrophilic silicone LEC growth and migration were seen, yet only in single LEC layers. Treatment with actinomycin D in the hydrophilic silicone lenses also reduced LEC growth. However, there were still sheets of LEC present on anterior and posterior parts of the capsules.

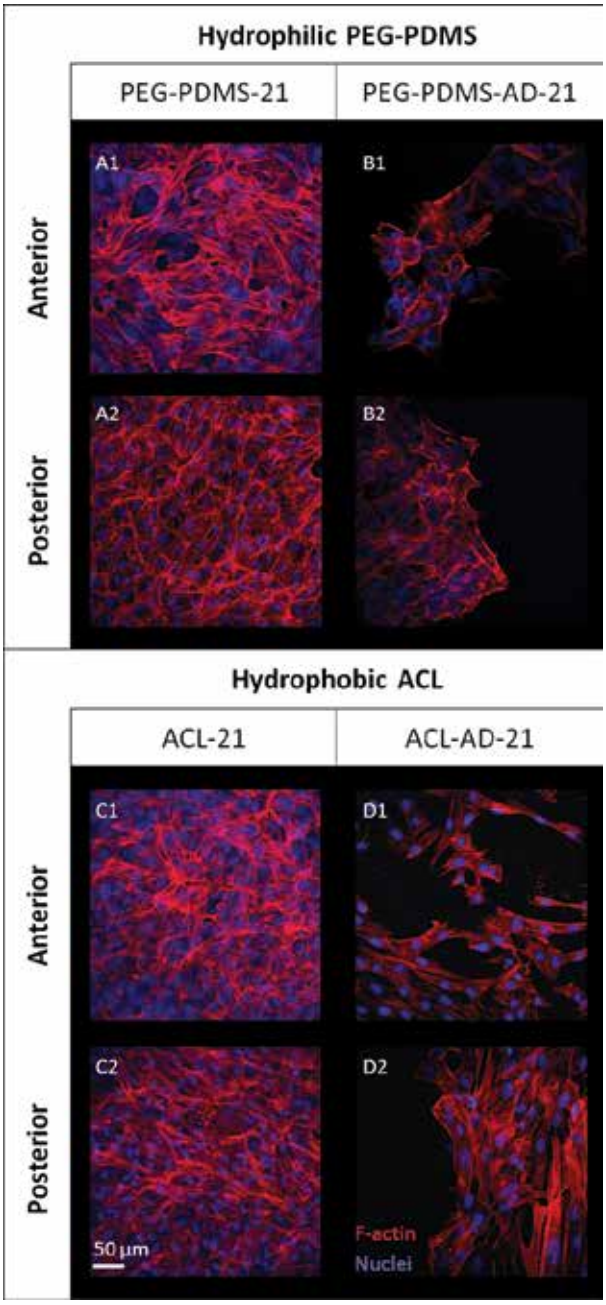


FIGURE 3. *Confocal images from the refilled lenses after three weeks of culturing. Of each refilling group one image from the anterior capsule (1) and one image from the posterior capsule (2) are shown. AD = Actinomycin D.*

No differences in lens thickness were found between groups in the viscoelasticity measurements. However, lenses refilled with hydrophobic ACL appeared to be thicker compared to lenses refilled with hydrophilic PEG-PDMS (Figure 2). When the three ACL refilled groups were combined and compared to the three PEG-PDMS groups, they were significantly thicker ($P = 0.032$). There were no differences in percentage relaxation between the groups. For the stiffness data, differences were found between the ACL-AD-21 and PEG-PDMS-AD-21 refilled lenses. Furthermore, a trend can be seen with higher stiffness values for the ACL refilled groups compared to the PEG-PDMS groups, and those differences were significant when the three ACL groups were combined and compared with the three PEG-PDMS groups ($P < 0.001$). For all the comparisons, no differences were found between groups with the same refilling material. Thus, no differences were seen between the ACL-0, ACL-21, and ACL-AD-21; and no differences between the three PEG-modified PDMS groups. Subsequently, the change in stiffness between day 0 and day 21 was not different in the ACL and PEG-PDMS groups.

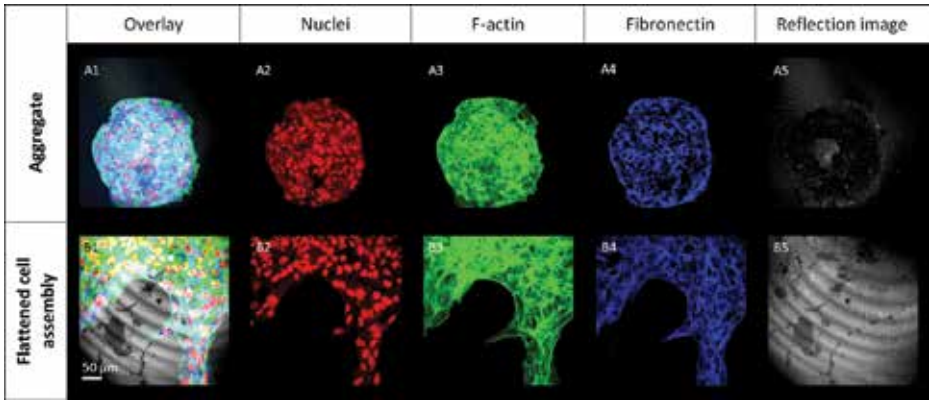


FIGURE 4. *Confocal images of LEC-B3 cells after 13 days of culturing on the hydrophilic silicone. The first series (A) presents a LEC aggregate on top of the PEG-PDMS and the second series (B) flattened LECs on the bottom of the wells plate. The LEC on the bottom of the wells plate originate from a cluster and now show some flattening with growth to the surrounding area on the wells plate, therewith displacing the viscous silicone side wards.*

Human LEC culture model

Both HLE-B3 cell cultures, on the hydrophilic silicone (Figure 4) and hydrophobic silicone (Figure 5) showed clustering of LECs in sphere shaped aggregates. No single LEC or confluent LEC layers were present on the material at all four time points after 2, 4, 7,

and 13 days on the hydrophilic silicone. After the first week of culture on the hydrophilic silicone, the LEC aggregates sank into the silicone material, flattened and showed some growth onto the bottom of the wells plate (Figure 4). However, the LECs remain separated from the surrounding silicone material as shown by the sharp delimitations on the confocal images. The LECs on the hydrophobic silicone showed similar patterns of flattened cell assembly after 5 days of culture (Figure 5).

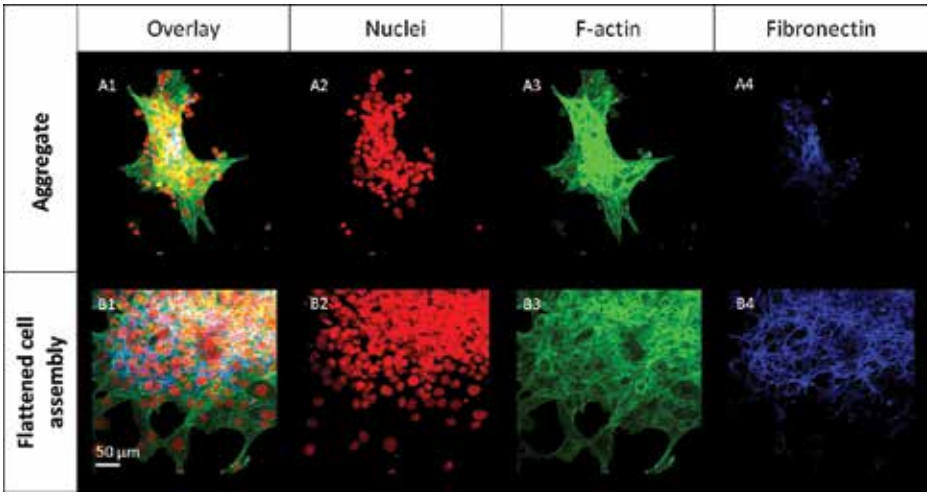


FIGURE 5. *Confocal images of LEC-B3 cells after 5 days of culturing on the hydrophobic silicone. The first series (A) presents a LEC aggregate and the second series (B) a flattened cell assembly of LECs.*

4.4 Discussion

The current study explores the effect of wettability on the development of CO and shows that CO formation in silicone refilled lenses is similar for hydrophilic and hydrophobic silicone polymers. Only small differences were seen on the confocal images after 21 days of lens culture, in which lenses refilled with the hydrophilic silicone showed less LEC growth. However, in *ex vivo* lens cultures with both refilling materials sheets of LECs were present after 3 weeks culture, and in the *in vitro* cell cultures on both materials LEC growth was present too. This indicates that there are small differences in the degree of LEC growth between the materials, but these differences are not relevant with regard to prevention of CO.

Other studies have examined the use of chemical agents to prevent CO in accommodative lens refilling (Koopmans et al., 2011, Koopmans et al., 2014, Nibourg et al., 2015a, Stachs et al., 2011, van Kooten et al., 2006), or investigated other aspects of silicones used in accommodative lens refilling, such as modulus, refractive index, and transparency (de Groot et al., 2001, de Groot et al., 2003, Hao et al., 2012). In our study, the combination of variation in wettability and treatment with actinomycin D did not lead to full prevention of CO. Compared to previous studies on actinomycin D treatment in lens refilling in *in vivo* monkey and rabbit models (Koopmans et al., 2014, Stachs et al., 2011), our *ex vivo* porcine lens model showed LEC growth already after 3 weeks of culture in both silicones. A combination of actinomycin D treatment with *in vivo* hydrophobic ACL silicone refilling in monkey lenses resulted in lenses which were able to accommodate for a maximal period of 6 months (Koopmans et al., 2014). The same treatment in rabbit lenses resulted in absent CO formation after 1 year and little CO formation after 3 years. These differences between models indicate that the development of CO varies between animal models and highlight the importance of experimental model selection in research for CO prevention (Wormstone and Eldred, 2015).

According to a previous study we would expect an increase in lens stiffness in accordance with the development of CO in the refilled lenses (Nibourg et al., 2015a). In the previous study we performed the viscoelasticity measurements after three months of lens culture, whereas in the current study lenses were only cultured for 3 weeks. Although we noticed LEC proliferation and migration to the posterior capsular bag after 21 days of culture, we did not measure an increase in lens stiffness with the viscoelasticity measurements. Three weeks is a relatively short period for LECs to proliferate and migrate, however we observed full CO formation in the lenses. We have selected this culture time for the present study since another experiment showed differences in LEC growth after 3 weeks of lens culture (Nibourg LM, et al. 2015c).

The differences in thickness between the lenses refilled with hydrophilic PEG-PDMS and hydrophobic ACL could be caused by variation in material properties. The ACL silicone has a lower viscosity compared with the PEG-PDMS silicone, which probably has resulted in a higher degree of filling of the ACL lenses. We also need to consider that these filling differences explain the stiffness differences between the ACL and PEG-PDMS refilled lenses.

The HLE-B3 cultures on the hydrophilic and hydrophobic silicones showed a similar repellence of LEC from silicone. There was no LEC growth on top of the silicone material,

and after seeding the cells they directly started the formation of aggregates. We noticed only some growth of LECs when the aggregates came into contact with the bottom of the wells plate. The LECs on the bottom of the plate showed sharp delineations indicating separation from the silicone material. The culture times of LECs on both silicones were not equal, with 13 days for the hydrophilic silicone and 5 days for the hydrophobic silicone. However, it can be assumed that longer culture times on the hydrophobic silicone would also have resulted in LEC repellence on the material.

The present study shows no relevant differences in CO formation caused by the use of hydrophilic or hydrophobic silicone polymers. This indicates that material wettability alone does not seem to be relevant for future applications of silicones in accommodation restoring techniques for cataract surgery.

References

Arshinoff S.A., Albiani D.A., Taylor-Laporte J., 2002. Intraocular pressure after bilateral cataract surgery using Healon, Healon5, and Healon GV. J. Cataract Refract. Surg. 28, 617-625.

Aslam T.M., Devlin H., Dhillon B., 2003. Use of Nd:YAG laser capsulotomy. Surv. Ophthalmol. 48, 594-612.

Berson F.G., Patterson M.M., Epstein D.L., 1983. Obstruction of aqueous outflow by sodium hyaluronate in enucleated human eyes. Am. J. Ophthalmol. 95, 668-672.

de Groot J.H., Spaans C.J., van Calck R.V., van Beijma F.J., Norrby S., Pennings A.J., 2003. Hydrogels for an accommodating intraocular lens. An explorative study. Biomacromolecules 4, 608-616.

de Groot J.H., van Beijma F.J., Haitjema H.J., Dillingham K.A., Hodd K.A., Koopmans S.A., Norrby S., 2001. Injectable intraocular lens materials based upon hydrogels. Biomacromolecules 2, 628-634.

Ge J., Wand M., Chiang R., Paranhos A., Shields M.B., 2000. Long-term effect of Nd:YAG laser posterior capsulotomy on intraocular pressure. Arch. Ophthalmol. 118, 1334-1337.

Haefliger E., Parel J.M., Fantes F., Norton E.W., Anderson D.R., Forster R.K., Hernandez E., Feuer W.J., 1987. Accommodation of an endocapsular silicone lens (Phaco-Ersatz) in the nonhuman primate. Ophthalmology 94, 471-477.

Hao X., Jeffery J.L., Le T.P., McFarland G., Johnson G., Mulder R.J., Garrett Q., Manns F., Nankivil D., Arrieta E., Ho A., Parel J.M., Hughes T.C., 2012. High refractive index polysiloxane as injectable, in situ curable accommodating intraocular lens. Biomaterials 33, 5659-5671.

Hao X., Jeffery J.L., Wilkie J.S., Meijs G.F., Clayton A.B., Watling J.D., Ho A., Fernandez V., Acosta C., Yamamoto H., Aly M.G., Parel J.M., Hughes T.C., 2010. Functionalised polysiloxanes as injectable, in situ curable accommodating intraocular lenses. Biomaterials 31, 8153-8163.

Kessler J., 1964. Experiments in Refilling the Lens. Arch. Ophthalmol. 71, 412-417.

Koopmans S.A., Terwee T., Barkhof J., Haitjema H.J., Kooijman A.C., 2003. Polymer refilling of presbyopic human lenses in vitro restores the ability to undergo accommodative changes. Invest. Ophthalmol. Vis. Sci. 44, 250-257.

Koopmans S.A., Terwee T., Glasser A., Wendt M., Vilupuru A.S., van Kooten T.G., Norrby S., Haitjema H.J., Kooijman A.C., 2006. Accommodative lens refilling in rhesus monkeys. Invest. Ophthalmol. Vis. Sci. 47, 2976-2984.

Koopmans S.A., Terwee T., Hanssen A., Martin H., Langner S., Stachs O., van Kooten T.G., 2014. Prevention of capsule opacification after accommodating lens refilling: pilot study of strategies evaluated in a monkey model. J. Cataract Refract. Surg. 40, 1521-1535.

Koopmans S.A., Terwee T., van Kooten T.G., 2011. Prevention of capsular opacification after accommodative lens refilling surgery in rabbits. Biomaterials 32, 5743-5755.

Lapčík L., Lapčík L., De Smedt S., Demeester J., Chabrecek P., 1998. Hyaluronan: preparation, structure, properties, and applications. Chem. Rev. 98, 2663-2684.

Marcantonio J.M., Syam P.P., Liu C.S., Duncan G., 2003. Epithelial transdifferentiation and cataract in the human lens. Exp. Eye Res. 77, 339-346.

Nibourg L.M., Sharma P.K., van Kooten T.G., Koopmans S.A., 2015a. Changes in lens stiffness due to capsular opacification in accommodative lens refilling. Exp. Eye Res. 134, 148-154.

Nibourg L.M., Gelens E., Kuijer R., Hooymans J.M.M., van Kooten T.G., Koopmans S.A., 2015b. Prevention of posterior capsular opacification. Exp. Eye Res. 136, 100-115.

Nibourg LM, Gelens E, de Jong M, Kuijer R, van Kooten TG and Koopmans SA, 2015c. Nanofiber-based hydrogels with extracellular matrix-based synthetic peptides for the prevention of capsular opacification. *Accepted for publication in Experimental Eye Research.*

Nishi O., Nishi Y., Chang S., Nishi K., 2014. Accommodation amplitudes after an accommodating intraocular lens refilling procedure: In vivo update. Journal of Cataract & Refractive Surgery 40, 295-305.

Nishi Y., Mireskandari K., Khaw P., Findl O., 2009. Lens refilling to restore accommodation. J. Cataract Refract. Surg. 35, 374-382.

Sharma P.K., Busscher H.J., Terwee T., Koopmans S.A., van Kooten T.G., 2011. A comparative study on the viscoelastic properties of human and animal lenses. Exp. Eye Res. 93, 681-688.

Stachs O., Langner S., Terwee T., Sternberg K., Martin H., Schmitz K.P., Hosten N., Guthoff R., 2011. In vivo 7.1 T magnetic resonance imaging to assess the lens geometry in rabbit eyes 3 years after lens-refilling surgery. J. Cataract Refract. Surg. 37, 749-757.

Steinert R.F., Puliafito C.A., Kumar S.R., Dudak S.D., Patel S., 1991. Cystoid macular edema, retinal detachment, and glaucoma after Nd:YAG laser posterior capsulotomy. Am. J. Ophthalmol. 112, 373-380.

Trinavarat A., Atchaneeyasakul L., Udompunturak S., 2001. Neodymium:YAG laser damage threshold of foldable intraocular lenses. J. Cataract Refract. Surg. 27, 775-780.

van Kooten T.G., Koopmans S., Terwee T., Norrby S., Hooymans J.M., Busscher H.J., 2006. Development of an accommodating intra-ocular lens--in vitro prevention of re-growth of pig and rabbit lens capsule epithelial cells. Biomaterials 27, 5554-5560.

Weeber H.A., van der Heijde R.G., 2007. On the relationship between lens stiffness and accommodative amplitude. Exp. Eye Res. 85, 602-607.

Wormstone I.M., Eldred J.A., 2015. Experimental models for posterior capsule opacification research. Exp. Eye Res. Epub.

Chapter 5

Effects of hyaluronan and sustained release of actinomycin D on capsular opacification after cataract surgery in a porcine eye model

Lisanne M. Nibourg, Theo G. van Kooten, Thom Terwee,
and Steven A. Koopmans

Abstract

Hyaluronan plays a role in signaling in the lens epithelial cells (LECs) via the CD44 cellular adhesion receptor. We aimed to investigate the influence of hyaluronan on LECs in porcine eyes by replacing the lens fibers with different solutions of hyaluronan in saline and in purified water. The effect of sustained release of actinomycin D on LECs was examined by dissolving actinomycin D in two solutions containing hyaluronan in purified water. Lenses refilled with hyaluronan solutions without actinomycin D all showed migration of LECs to the posterior capsule and expression of αSMA after three weeks of culture. LECs from lenses refilled with hyaluronan dissolved in purified water appeared in a more normal morphology compared to LECs from the saline groups. The concentration of hyaluronan showed dose-dependent trends on LEC migration and transdifferentiation. Sustained release of actinomycin D dissolved in the hyaluronan solutions resulted in a total absence of LEC on the capsular bag in all lenses. The current study demonstrates that hyaluronan influences the LEC response in a refilled porcine lens model by changing concentrations and osmolarity. Furthermore, sustained release of actinomycin D from hyaluronan solutions can prevent capsular opacification.

5.1 Introduction

Hyaluronan (sodium hyaluronate) is a polysaccharide with special chemical properties such as strong hydration and viscoelasticity that depends on the size of the molecule (Lapčík et al., 1998). Because of these characteristics, it is widely used in cataract surgery as a solution that maintains the anterior chamber during capsulorhexis and lens implantation (Arshinoff, 1986). Interestingly, hyaluronan also plays a role in signaling in the lens epithelial cells (LECs) via the CD44 receptor. This signaling is initiated by tissue trauma following cataract surgery and may result in development of capsular opacification (CO) (Eldred et al., 2011, Lopez-Novoa and Nieto, 2009). CO is a common complication of cataract surgery causing a diminished visual acuity as a consequence of proliferation, migration, and transdifferentiation of LECs on the lens capsule in the visual axis (Marcantonio and Vrensen, 1999). The prevention of CO is important for present and new intraocular lenses such as accommodative lenses. Many artificial accommodative lens concepts use the elasticity of the lens capsular bag to transform the ciliary muscle

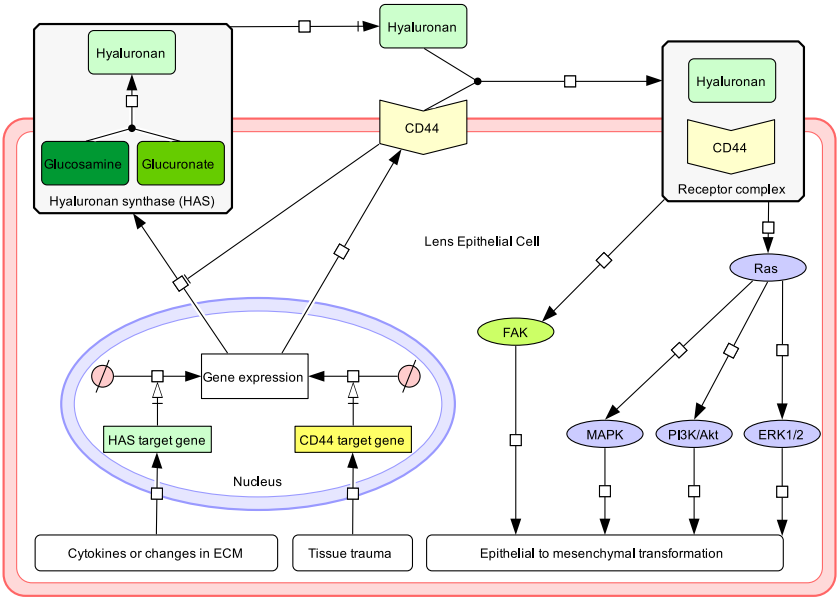


FIGURE 1. *Schematic overview of hyaluronan/CD44 associated signaling pathways in LECs. Tissue trauma initiates upregulation of CD44. The glycosaminoglycan hyaluronan is formed out of the substrates N-acetyl-D-glucosamine and D-glucuronate by hyaluronan synthases (HAS) in response to cytokines or changes in the extracellular matrix (ECM) and is subsequently released into the ECM. Hyaluronan can bind to the CD44 receptor in order to initiate signaling associated with EMT in the lens epithelium. FAK: focal adhesion kinase; MAPK: mitogen-activated protein kinase; PI3K/Akt: phosphatidylinositide 3-kinase/Akt; ERK1/2: extracellular signal-regulated kinase; Arrows with black arrowhead: influence cellular processes; Arrows with black arrowhead and stripe: transport or release of molecule; Arrows with white arrowhead and stripe: trigger for specific process. Figure made with CellDesigner (Funahashi et al., 2003).*

activity into a shape-change of the artificial lens. This is the case in accommodative lens refilling, where the lens content is removed from the capsular bag during cataract surgery and replaced by a soft transparent injectable polymer restoring accommodation of the presbyopic eye (Nishi et al., 2009). Since CO may cause stiffening and opacification of the capsular bag this limits visual acuity and the amplitude of accommodation which indicates the interest of CO prevention for the development of new techniques in cataract surgery.

In CO, the process of proliferation, migration, and transdifferentiation of LECs is also known as epithelial to mesenchymal transformation (EMT) (de Iongh et al., 2005). Signaling pathways of the growth factor TGF-β play important roles in EMT, but many

other signaling molecules have been found to influence LEC behavior (Nibourg et al., 2015b, Wormstone et al., 2009). One of these pathways associated with TGF-β signaling is the CD44/hyaluronan pathway (Figure 1). CD44 is a cell membrane receptor for cell adhesion which can interact with different signaling molecules, like growth factors, matrix metalloproteinases, and hyaluronan, and is upregulated after tissue trauma (Acharya et al., 2008). CD44 and hyaluronan were detected in human anterior capsules obtained during cataract surgery (Nishi et al., 1997, Saika et al., 1998), and canine LECs treated with different concentrations of hyaluronan showed a dose-dependent increase in CD44 expression and increased LEC migration (Chandler et al., 2012). In previous *in vivo* experiments in rhesus monkeys aimed at accommodative lens refilling we found that lens refilling with hyaluronan after a short treatment of the LEC with the cytostatic actinomycin D resulted in a clear lens in the treated animal (Koopmans et al., 2014). Refilling the lens capsular bag with a silicone polymer after treatment with actinomycin D resulted in the development of CO (Koopmans et al., 2006). Therefore, with the current study we aimed to investigate the influence of hyaluronan and actinomycin D on LEC behavior and CO formation after lens refilling in porcine eyes with different hyaluronan solutions.

5.2 Material and Methods

Lenses

Twenty fresh natural porcine *(Sus domesticus)* eyes were obtained from the local slaughterhouse. The eyes were from pigs with an age of approximately six months. The lens content was removed, and the lenses were refilled with different concentrations of hyaluronan (molecular weight 4 x 10^6 Da, Abbott Medical Optics, Uppsala, Sweden) dissolved in saline (NaCl, 300 mOsm/L) or purified water (milli-Q, EMD Millipore Corporation, Billerica, USA), according to the surgical refilling procedure below. In five eyes, the cytotoxic agent actinomycin D (Sigma-Aldrich Corporation, St. Louis, USA) was added to the hyaluronan. The concentrations used for the hyaluronan dissolved in saline were: 5, 10, and 20 mg/mL. Concentrations for the hyaluronan in purified water were: 10 and 20 mg/mL. For the hyaluronan in purified water with the addition of 10 µg/mL actinomycin D concentrations were: 10 and 20 mg/mL.

Hyaluronan preparation

First, 500 mL buffer was prepared, containing 4250 mg sodium chloride, 140 mg disodium hydrogen phosphate dihydrate, and 20 mg sodium dihydrogen phosphate dihydrate dissolved in purified water or saline and filter sterilized. The hyaluronan fibers were stored in the freezer at -20 °C. For preparation of the hyaluronan gel, the fibers were weighed and subsequently washed with 70% ethanol for sterilization after which they were laid out to dry in a flow cabinet for approximately 1 hour. Then the buffer was added to the fibers and the mix was put at 4°C for a minimum of 12 hours, after which a homogeneous gel was formed. Next the gel was transferred to syringes. In five preparations, 10 µg/mL actinomycin D was dissolved in the purified water before adding this solution to the hyaluronan fibers.

Surgical refilling procedure and extraction of the lens

The surgical lens refilling procedure is based on a method previously described (Koopmans et al., 2003, Koopmans et al., 2006, Koopmans et al., 2014, Nibourg et al., 2015a). In short, the lens contents were aspirated through a capsulorhexis of 1.0-1.5 mm diameter. No efforts were made to remove the lens epithelial cell layer intentionally. Then, a 3.0 mm diameter capsular plug was inserted to close the capsular bag, and the hyaluronan solution was injected until it started to leak around the plug and the capsular bag was completely filled. The refilled lenses were extracted from the eye. First the cornea was cut off with scissors containing sharp curved blades. Then the iris was removed and the zonules were cut using blunt Westcott scissors. The attached vitreous was removed by lifting the lens and cutting vitreous strands, and the lens was placed in a culture medium.

Culturing method

The lenses were kept in culture medium consisting of minimal essential medium (MEM) supplemented with 12% fetal bovine serum (FBS), 2 mM GlutaMAX™-I, 1 mM sodium pyruvate, and 500 Units/mL penicillin – 500 µg/mL streptomycin – 1.25 µg/mL amphotericin B (all Life Technologies Ltd, Paisley, UK). The lenses were cultured for three weeks in a 37 °C 5% CO_2 incubator. The medium was changed twice a week.

Staining and imaging of lens epithelial cells

After three weeks of culturing, the lenses were fixated with 3.7% paraformaldehyde for 30 minutes. After permeabilization in 0.5% Triton X-100 in PBS for 15 minutes, the

LECs were stained with 2 µg/mL TRITC-labeled phalloidin and 4 µg/mL 4',6-diamidino-2-phenylindole (DAPI) dissolved in PBS containing 1% bovine serum albumin (PBSA) for 30 minutes. At this stage staining involving antibodies could not be performed as the lens capsular bag was kept intact and the capsule is not permeable for large proteins. Microscopic observation was carried out within one week after fixation, until then the filled lenses were stored in PBS. We used the Leica TCS SP2 Confocal Laser Scanning Microscope (Leica Microsystems, Wetzlar, Germany) for imaging.

The next step was immunofluorescence staining for alpha smooth muscle actin (αSMA), which is a marker for myofibroblasts (Marcantonio et al., 2003). For this the hyaluronan was removed from the lenses by removing the plug and emptying the capsule, therewith allowing free diffusion of antibodies used in the staining of αSMA. Subsequently, the lens capsules were blocked in 5% bovine serum albumin (BSA) in PBS for 30 minutes. The lens capsules were then incubated with a 1:100 dilution of primary antibody mouse-anti-human αSMA (Sigma-Aldrich Co, St. Louis, USA) in PBSA for 1 hour. Next, lenses were washed three times 10 minutes in PBSA and incubated for 1 hour in the secondary antibody, 1:100 diluted FITC-labeled goat-anti-mouse IgG (Jackson ImmunoResearch Europe Ltd, Suffolk, UK) in PBSA. Lenses were again washed two times 5 minutes in PBSA, and were stored in PBSA until microscopic observation. For microscopic observation lens capsular bags were mounted on object slides, covered with citifluor (Citifluor Ltd, London, UK) and cover-slipped.

From each lens, nine images were made using the confocal microscope. Six images were from the intact lens (three posterior, three anterior) and three images from the mounted capsules after immunofluorescence staining for αSMA. From these images the total number of cells and the number of αSMA stained cells were counted using ImageJ (Schneider et al., 2012). Only cells showing a complete cytoplasmatic αSMA staining were counted, providing the best possible overview of functional αSMA expression and excluding background staining. For each image, the percentage of cells showing αSMA expression was calculated from the total number of cells.

Statistical analyses

The Statistical Package for the Social Sciences (IBM SPSS Statistics 20) was used to analyze the data. Differences between groups in cell count and αSMA staining were evaluated using Wilcoxon rank-sum tests and analyses of variance (ANOVA) with Games-Howell post-hoc analyses. P values ≤ 0.05 were considered statistically significant.

5.3 Results and discussion

Lenses and lens epithelial cell response

Twenty-four fresh natural porcine eyes were obtained from the local slaughterhouse. The eyes came from pigs of the same size, breed, and age, providing a homogenous group of lenses for the experiments and minimizing individual differences. Because of dropout, 19 lenses divided into seven groups were left (Table 1).

TABLE 1. *Overview of used materials and results from the tested hyaluronan combinations.*

	Materials			Results	
	Composition hyaluronan	N	Concentration hyaluronan	Cell count (± SD)*	% αSMA (± SD)
1	Normal saline	2	5 mg/mL	298 (190)	49.02 (11.36)
2	Normal saline	3	10 mg/mL	667 (1167)	29.25 (30.65)
3	Normal saline	3	20 mg/mL	1320 (1058)	20.03 (15.82)
4	Purified water	4	10 mg/mL	1644 (2280)	30.08 (24.60)
5	Purified water	2	20 mg/mL	768 (597)	24.80 (12.36)
6	Purified water + actinomycin D	2	10 mg/mL	0 (0)	0.00 (0.00)
7	Purified water + actinomycin D	3	20 mg/mL	0 (0)	0.00 (0.00)

αSMA= alpha-smooth muscle actin. * Cell count within a field of view of 750 x 750 µm.

The lenses refilled with hyaluronan without actinomycin D all showed migration of LEC to the posterior capsule (Figure 2, images 2A2-2E2) and expression of αSMA (Table 1) after three weeks of culture. The cell morphology appeared similar for the three concentrations of saline (Figure 2, A, B, and C) or the two concentrations of purified water (Figure 2, D and E) and also cell counts were not different (Table 1, P-values ≥ 0.172) (further discussed in section 5.3 page 125). There were differences between the cell morphology of the LEC from the purified water groups and the saline groups, in which in the saline groups the posterior capsule was fully covered by LECs and in the purified water groups the LECs appeared in a more clustered pattern. Furthermore, the shape of the LECs was different in the saline groups compared to purified water groups (further discussed in section 5.3 page 123). The addition of actinomycin D to the hyaluronan in purified water resulted in total absence of viable LECs on both the anterior and posterior capsule (Figure

2, F and G). In some of the chemically treated lenses we observed clusters of residual actin fibers located on the inside of the lens capsule (as seen in images 2F1 and 2G2).

Hyaluronan in saline solutions vs. purified water solutions

As discussed above, differences were seen in LEC morphology between the groups refilled with hyaluronan dissolved in saline and purified water (Figure 2). The saline groups showed a more irregular LEC pattern compared to the purified water groups, which contained round shaped LECs. Furthermore, the posterior capsules from the purified water groups were not totally covered by LEC and showed a more clustered pattern (images 2D2-2E2). Stretched transformed LEC were only present in the saline 20 mg/mL group (image 2C1). However, when the cell count and αSMA expression of the saline groups were compared to the purified water groups there were no significant differences ($P = 0.347$ and $P = 0.770$, respectively). Examples of microscopy images with αSMA staining in the hyaluronan groups with 10 mg/mL are given in Figure 3 (images 3A2-3B2). The images from the saline refilled lens (3A2) and the water refilled lens (3B2) both show cytoplasmatic αSMA staining of single LEC with a similar percentage of αSMA positive cells.

Next to this, we observed differences in the viscosity and the handling of the materials. The hyaluronan dissolved in saline was more viscous compared to the same concentration in purified water. That also explains why we were not able to include a group refilled with 5 mg/mL hyaluronan in purified water, because the low viscosity of this composition caused leakage through the plug and resulted in empty lens capsules within two days of culturing. Initially, we aimed to examine concentrations of 2.5 mg/mL hyaluronan as well, but for purified water and saline the viscosity was too low to maintain the capsular bag in culture. A possible explanation for these differences in viscosity between the saline and purified water solutions could be that saline is isotonic compared to hypotonic purified water, and therefore the hyaluronan solutions with saline displayed a higher viscosity (Rand and Lacombe, 1964).

Tonicity and osmolarity of viscoelastics used for cataract surgery are important for proper preservation of the cornea (Dick et al., 2000). The osmolarity of corneal tissue is 305 mOsmol/kg and 300 mOsmol/kg for the aqueous (Dick et al., 2000), compared to initially 308 mOsmol/L (equivalent to ± 306 mOsmol/kg) for saline (NaCl 0.9%, Baxter, Utrecht, the Netherlands) and 0 mOsmol/L for purified water before mixing with the hyaluronan fibers. Thus based on these numbers the hyaluronan in saline used in our study had the preferred osmolarity for maintaining corneal integrity. However, our study

showed the best LEC morphology in the groups refilled with hyaluronan in purified water. Treatment of the capsular bag with hypo-osmotic deionized, distilled water has been shown to reduce capsular opacification post-cataract surgery (Rekas et al., 2013), presumably by causing cytolysis of LECs (Crowston et al., 2004). Although the purified water solution, used for either dissolving hyaluronan or for treatment of the LEC, will change due to diffusion and osmosis of small molecules and ions, the observed effects of hypo-osmotic environments on the LEC response indicate that such an approach can be incorporated in methods for CO prevention.

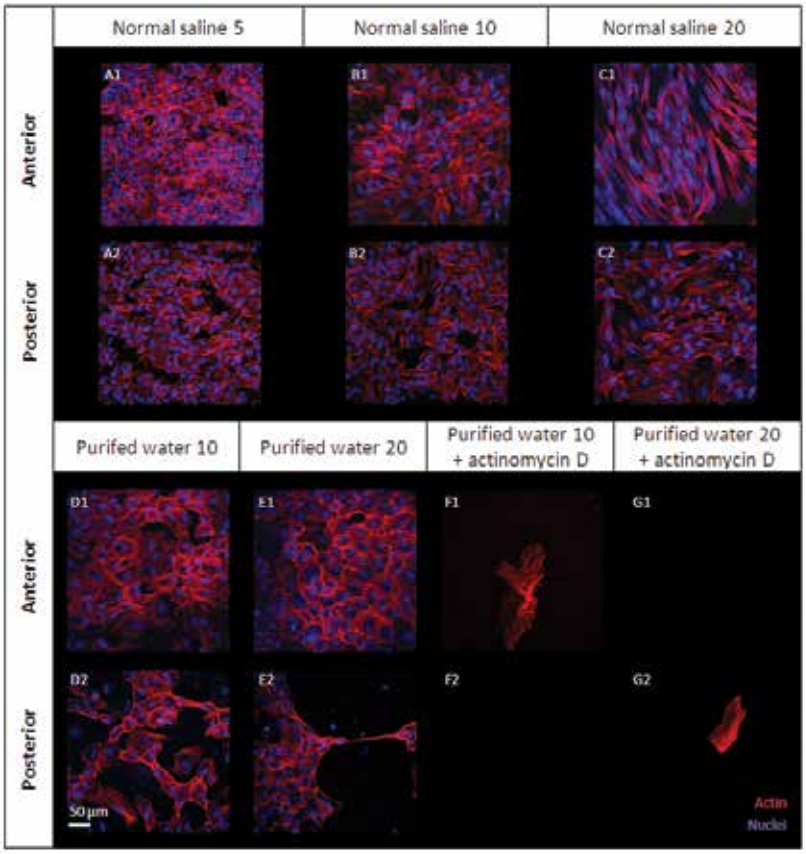


FIGURE 2. *Microscopic images from lenses refilled with the different concentrations of hyaluronan. The unit for the concentrations of hyaluronan is mg/mL. It can be seen that the groups without actinomycin D treatment show LEC on the posterior capsule (images 2A2-2E2). The groups with actinomycin show a total absence of LEC (images 2F-2G).*

Effect of hyaluronan concentrations on lens epithelial cells

Our data only showed a slight effect of hyaluronan concentration on the LEC response in the saline group (Figure 2, images 2A-2C). We observed some stretched LECs in 20 mg/mL saline refilled lenses but the expression of αSMA in this group was also lower compared to the 5 mg/mL saline group (P = 0.016).Although no significant differences were found in cell count between those groups (P = 0.172), it seems that the high hyaluronan concentration resulted in higher cell densities and a more irregular cell layer. In the purified water groups no differences were found in αSMA expression or cell count between 10 and 20 mg/mL concentrations (P = 0.996 and P = 0.864, respectively). Unfortunately, we were not able to create a stable refilling with lower concentrations of hyaluronan in purified water as described earlier in this paper. Our results showed dose-dependent trends of hyaluronan on LEC migration and transdifferentiation. Canine capsules treated with 12 and 20 mg/mL hyaluronan demonstrated a dose-dependent increase in LEC migration (Chandler et al., 2012). In that study, commercially available viscoelastics were used which contain different salts and have an osmolarity between 300-350 mOsmol/kg (Acrivet 1.2% and 2%, Hennigsdorf, Germany).

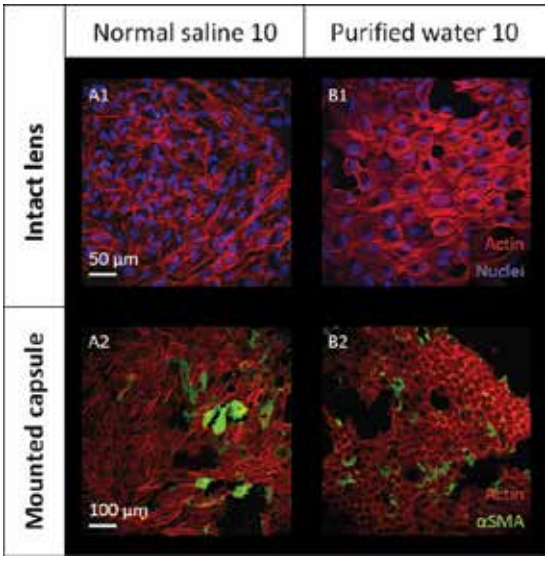


FIGURE 3. *Microscopic images with an example of αSMA staining from posterior capsules of lenses refilled with 10 mg/mL hyaluronan in saline and in purified water. The images from the saline refilled lens (3A2) and the water refilled lens (3B2) both show cytoplasmatic staining of single LEC with a similar percentage of αSMA stained cells.*

Effect of hyaluronan with sustained release of actinomycin D

Treatment with 10 µg/mL actinomycin D dissolved in hyaluronan with purified water resulted in an absence of LECs on both the anterior and posterior capsule (Figure 2). Consequently, those lenses did not stain with αSMA, and significant differences were found in αSMA expression between the solutions with and without actinomycin D treatment (Table 1, $P < 0.001$). Compared to other studies of actinomycin lens capsule treatment (Koopmans et al., 2006, Koopmans et al., 2014, Nibourg et al., 2015a, Sternberg et al., 2010, van Kooten et al., 2006), this is the first study where a total absence of LEC on the lens capsule in multiple samples was found. Our outcome confirms earlier results in which a clear lens was found after actinomycin treatment followed by hyaluronan refilling in a rhesus monkey eye nine months post-surgery (Koopmans et al., 2014). Contrastingly, other studies in which actinomycin treatment was followed by refilling of the capsular bag with a silicone polymer showed that actinomycin slowed down the formation of CO, but did not result in full prevention (Koopmans et al., 2006, Nibourg et al., 2015a). Even when porcine lens capsules were treated with high doses actinomycin dissolved in purified water (50 µg/mL for 1 h) there were LEC situated in the equatorial region after three months of culture (Nibourg et al., 2015a). Thus, it may be that refilling the capsular bag with hyaluronan after chemical treatment provides a proper environment to prevent LEC growth. However, it is more likely that the absence of LEC in our study is caused by providing a sustained release of actinomycin D since it was dissolved in the hyaluronan. In contrast, in previous studies the actinomycin was administered for a certain period after which the capsular bag was irrigated and refilled with another material. So we now demonstrate that with a sustained release of actinomycin D total prevention of CO can be accomplished in an *ex vivo* porcine eye model. As positive effects in animal models are no guarantee for clinical success, further research is required to determine the effects in *in vivo* animal models in order to finally work to CO prevention in patients. The contribution of this study has been to assess the role of hyaluronan in CO formation and to explore the effects of sustained release of actinomycin D for CO prevention. Long-term exposure to actinomycin will increase the risk for diffusion through the capsular bag and consequently increases the risk for toxic effects to other ocular tissues (Kastner et al., 2013). Thus for future applications toxic effects resulting from the permeability of actinomycin D through the capsule need to be solved in order to safely use this agent in the eye, whereas this is a relevant issue for all pharmaceutics used within the lens capsular bag.

5.4 Conclusions

In this study we showed that hyaluronan can influence the LEC response after refilling porcine lens capsules with compositions of hyaluronan in saline and purified water. Refilling of capsules with hyaluronan dissolved in purified water resulted in LEC with a normal morphology, and our results showed a dose-dependent trend of hyaluronan on LEC migration and transdifferentiation in the saline groups. Furthermore, treatment with sustained release of actinomycin D dissolved in the hyaluronan resulted in a total absence of LEC on the capsular bag and showed that sustained release of actinomycin D is a potential method for CO prevention.

Acknowledgements

The authors want to thank Simone Nibourg for the assistance with processing of the data.

References

Acharya P.S., Majumdar S., Jacob M., Hayden J., Mrass P., Weninger W., Assoian R.K., Pure E., 2008. Fibroblast migration is mediated by CD44-dependent TGF beta activation. J. Cell. Sci. 121, 1393-1402.

Arshinoff S.A., 1986. Viscoelastic substances: their properties and use when placing an IOL in the capsular bag. Curr can Ophthalmic Prac. 4, 64-65.

Chandler H.L., Haeussler D.J.,Jr, Gemensky-Metzler A.J., Wilkie D.A., Lutz E.A., 2012. Induction of posterior capsule opacification by hyaluronic acid in an ex vivo model. Invest. Ophthalmol. Vis. Sci. 53, 1835-1845.

Crowston J.G., Healey P.R., Hopley C., Neilson G., Milverton E.J., Maloof A., 2004. Water-mediated lysis of lens epithelial cells attached to lens capsule. J. Cataract Refract. Surg. 30, 1102-1106.

de Iongh R.U., Wederell E., Lovicu F.J., McAvoy J.W., 2005. Transforming growth factor-beta-induced epithelial-mesenchymal transition in the lens: a model for cataract formation. Cells Tissues Organs 179, 43-55.

Dick H.B., Augustin A.J., Pfeiffer N., 2000. Osmolality of various viscoelastic substances: comparative study. J. Cataract Refract. Surg. 26, 1242-1246.

Eldred J.A., Dawes L.J., Wormstone I.M., 2011. The lens as a model for fibrotic disease. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 366, 1301-1319.

Funahashi A., Morohashi M., Kitano H., Tanimura N., 2003. CellDesigner: a process diagram editor for gene-regulatory and biochemical networks. Biosilico 1, 159-162.

Kastner C., Lobler M., Sternberg K., Reske T., Stachs O., Guthoff R., Schmitz K.P., 2013. Permeability of the Anterior Lens Capsule for Large Molecules and Small Drugs. Curr. Eye Res. 38, 1057-63.

Koopmans S.A., Terwee T., Barkhof J., Haitjema H.J., Kooijman A.C., 2003. Polymer refilling of presbyopic human lenses in vitro restores the ability to undergo accommodative changes. Invest. Ophthalmol. Vis. Sci. 44, 250-257.

Koopmans S.A., Terwee T., Glasser A., Wendt M., Vilupuru A.S., van Kooten T.G., Norrby S., Haitjema H.J., Kooijman A.C., 2006. Accommodative lens refilling in rhesus monkeys. Invest. Ophthalmol. Vis. Sci. 47, 2976-2984.

Koopmans S.A., Terwee T., Hanssen A., Martin H., Langner S., Stachs O., van Kooten T.G., 2014. Prevention of capsule opacification after accommodating lens refilling: pilot study of strategies evaluated in a monkey model. J. Cataract Refract. Surg. 40, 1521-1535.

Lapčík L., De Smedt S., Demeester J., Chabrecek P., 1998. Hyaluronan: preparation, structure, properties, and applications. Chem. Rev. 98, 2663-2684.

Lopez-Novoa J.M., Nieto M.A., 2009. Inflammation and EMT: an alliance towards organ fibrosis and cancer progression. EMBO Mol. Med. 1, 303-314.

Marcantonio J.M., Syam P.P., Liu C.S., Duncan G., 2003. Epithelial transdifferentiation and cataract in the human lens. Exp. Eye Res. 77, 339-346.

Marcantonio J.M., Vrensen G.F., 1999. Cell biology of posterior capsular opacification. Eye (Lond) 13, 484-488.

Nibourg L.M., Sharma P.K., van Kooten T.G., Koopmans S.A., 2015a. Changes in lens stiffness due to capsular opacification in accommodative lens refilling. Exp. Eye Res. 134, 148-154.

Nibourg L.M., Gelens E., Kuijer R., Hooymans J.M.M., van Kooten T.G., Koopmans S.A., 2015b. Prevention of posterior capsular opacification. Exp. Eye Res. 136, 100-115.

Nishi O., Nishi K., Akaishi T., Shirasawa E., 1997. Detection of cell adhesion molecules in lens epithelial cells of human cataracts. Invest. Ophthalmol. Vis. Sci. 38, 579-585.

Nishi Y., Mireskandari K., Khaw P., Findl O., 2009. Lens refilling to restore accommodation. J. Cataract Refract. Surg. 35, 374-382.

Rand P.W., Lacombe E., 1964. Hemodilution, Tonicity, and Blood Viscosity. J. Clin. Invest. 43, 2214-2226.

Rekas M., Klus A., Kosatka M., 2013. Sealed-capsule irrigation with distilled deionized water to prevent posterior capsule opacification--prospective, randomized clinical trial. Curr. Eye Res. 38, 363-370.

Saika S., Kawashima Y., Miyamoto T., Okada Y., Tanaka S., Yamanaka O., Ohnishi Y., Ooshima A., Yamanaka A., 1998. Immunolocalization of hyaluronan and CD44 in quiescent and proliferating human lens epithelial cells. J. Cataract Refract. Surg. 24, 1266-1270.

Schneider C.A., Rasband W.S., Eliceiri K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671-675.

Sternberg K., Terwee T., Stachs O., Guthoff R., Lobler M., Schmitz K.P., 2010. Drug-induced secondary cataract prevention: experimental ex vivo and in vivo results with disulfiram, methotrexate and actinomycin D. Ophthalmic Res. 44, 225-236.

van Kooten T.G., Koopmans S., Terwee T., Norrby S., Hooymans J.M., Busscher H.J., 2006. Development of an accommodating intra-ocular lens--in vitro prevention of re-growth of pig and rabbit lens capsule epithelial cells. Biomaterials 27, 5554-5560.

Wormstone I.M., Wang L., Liu C.S., 2009. Posterior capsule opacification. Exp. Eye Res. 88, 257-269.

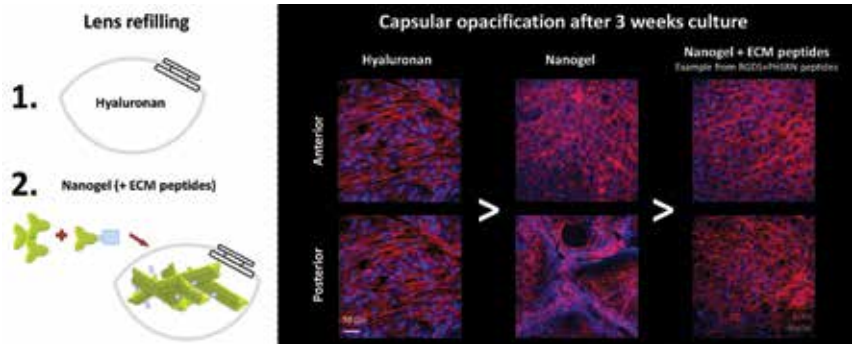
Chapter 6

Nanofiber-based hydrogels with extracellular matrix-based synthetic peptides for the prevention of capsular opacification

Lisanne M. Nibourg, Edith Gelens, Menno R. de Jong, Roel Kuijer, Theo G. van Kooten, and Steven A. Koopmans

This chapter is an edited version of the manuscript: Nibourg L.M., Gelens E., de Jong M.R., Kuijer R., van Kooten T.G., Koopmans S.A., 2015. Nano-fiber based hydrogels with extracellular matrix-based synthetic peptides for the prevention of capsular opacification. Experimental Eye Research, Epub. (Re-use permitted)

Graphical abstract



Abstract

Nanofiber-based hydrogels (nanogels) with different, covalently bound peptides were used as an extracellular environment for lens epithelial cells (LECs) in order to modulate the capsular opacification (CO) response after lens surgery in a porcine eye model. Lenses were divided into 15 groups (n=4 per group), the lens content was removed and the empty capsules were refilled with nanogel without peptides and nanogels with 13 combinations of 5 different peptides: two laminin-derived, two fibronectin-derived, and one collagen IV-derived peptide representing cell adhesion motifs. A control group of 4 lenses was refilled with hyaluronan. After refilling, lenses were extracted from the porcine eye and cultured for three weeks. LECs were assessed for morphology and alpha smooth muscle actin (αSMA) expression using confocal laser scanning microscopy. Compared to hyaluronan controls, lenses filled with nanogel had less CO formation, indicated by a lower αSMA expression (P=0.004). Microscopy showed differences in morphological cell response within the nanogel refilled groups. αSMA expression in these groups was highest in lenses refilled with nanogel without peptides (9.54±11.29%). Overall, LEC transformation is reduced by the presence of nanogels and the response is improved even further by incorporation of extracellular matrix peptides representing adhesion motifs. Thus, nanomaterials targeting biological pathways, in our case interactions with integrin signaling, are a promising avenue toward reduction of CO. Further research is needed to optimize nanogel-peptide combinations that fully prevent CO.

6.1 Introduction

Accommodative lens refilling (Koopmans et al., 2003, 2006, 2014) involves replacement of the lens with a silicone polymer in an otherwise intact lens capsule, enabling accommodation in presbyopic lenses. For the development of these injectable accommodating lenses, capsular opacification (CO) is a major problem, since these lenses require an intact and clear lens capsule (van Kooten et al., 2006). Nanogels, directing cell differentiation, could form a layer between the inserted lens refilling material and the surface of the capsular bag for the prevention of CO.

Capsular opacification is a common complication of cataract surgery and results in a diminished visual acuity as a consequence of proliferation, migration, and transdifferentiation of lens epithelial cells (LECs) in the visual axis (Marcantonio and Vrensen, 1999). This process of transdifferentiation of LECs to myofibroblasts is also known as epithelial to mesenchymal transformation (EMT) (de Iongh et al., 2005). TGF-β signaling pathways play important roles in EMT and CO development, but over the past decades many other signaling molecules have been found to influence LEC behavior (Nibourg et al., 2015b, Wormstone et al., 2009). Knowledge of underlying biological processes of the development of CO has changed the focus in research aimed at CO prevention. Nowadays this focus is more on agents that can interfere with these cellular processes. Rapid advances in material sciences also provide novel opportunities in research for CO prevention. Nanotechnology has recently been used in various ocular applications, including studies on CO prevention (Guha et al., 2013, Huang et al., 2013, Wang et al., 2013, Zhang et al., 2013).

In this study we developed an organ-culture model using porcine eye lenses in which, after surgical removal of the natural lens fibers, nanofiber-based hydrogels (nanogels) with attached signaling peptides were injected that by interacting with LECs should prevent EMT and therewith the formation of CO. The peptides were chosen according to their potential to influence integrin signaling pathways in LECs and were derived from the extracellular matrix (ECM) components laminin (IKVAV and YIGSR), fibronectin (RGDS and PHSRN), and collagen IV (DGEA) (Linnola et al., 2000, Olivero and Furcht, 1993). Binding of cells to components of the ECM is mediated by integrin receptors in the cell membrane. Integrin signaling is a cellular process involved in the development of CO (Mamuya et al., 2014, Walker and Menko, 2009). Most members of the integrin family have been identified in LECs (McLean et al., 2005, Worthington et al., 2011, Zhang et al.,

2000). Integrins can bind a large variety of ligands on ECM components and other molecules involved in cellular signaling (Walker and Menko, 2009, Wederell and de Iongh, 2006). After cataract surgery, the extracellular environment of LECs is changed. Changes in integrin-ECM interactions are associated with transdifferentiation of LECs, and integrins were found to be activators for TGF-β, which is present in the latent form in the ECM (Dawes et al., 2007, Mamuya et al., 2014, Worthington et al., 2011). These processes result in the development of CO (Mamuya et al., 2014, Walker and Menko, 2009).

For the current study we injected the whole capsule with nanogel until complete filling, since we aimed at monitoring interactions between LECs and nanogel to explore the possibilities for modulation of the capsular opacification response after lens surgery.

6.2 Material and Methods

Experimental setup

Fresh natural porcine *(Sus domesticus)* eyes were obtained from the local slaughterhouse. All eyes were from pigs with an age of approximately six months. The lens content was removed, and the lenses were refilled with different nanogel materials or with a hyaluronan control (sodium hyaluronate, Healon 10 mg/ml; Abbot Medical Optics, Uppsala, Sweden), according to the surgical refilling procedure described below. All refilling experiments were performed at n=4, except for the experiments with the YIGSR-nanogel (n=3) due to material shortage at the moment of the surgery. After refilling of the lens capsule, the lenses were extracted and cultured for three weeks.

Nanofiber-based hydrogel assembly and preparation

The nanofiber-based hydrogel consists of fibers of a low molecular weight hydrogelator (LMWG) (Brizard et al., 2008, Cui et al., 2010). In Figure 1 the structure of LMWG as well as a schematic representation of nanogel formation by self-assembly are shown. The synthesis of LMWG has previously been described by van Bommel et al. (2005). Using this process LMWG was synthesized in-house and characterized by 1H-NMR and HPLC-MS (+95% purity). LMWG was functionalized with a maleimide moiety using N-succinimidyl 3-maleimidopropionate (TCI, +95%) and subsequently reacted with cysteine-containing peptides (custom made at Think Peptides, >95% purity) through a process described

in detail by Tuin et al. (2015; *manuscript in preparation*) to obtain the building blocks (characterized by 1H-NMR and HPLC-MS) for the different nanogel-peptide composites (next subsection). Self-assembly of LMWG and LMWG-nanopeptides into fibers and subsequent gel formation was achieved by dissolution of the appropriate mixture of LMWG and peptide derivatives (Nano Fiber Matrices B.V., Groningen, The Netherlands) in a mixture of hydrochloric acid (0.18 M HCl, prepared from 1.0 M HCl (J.T. Baker, analytical grade) diluted with distilled water (Boom)) and saline solution (NaCl (ESCO), dissolved in distilled water (Boom); solutions were sterilized prior to use by filtration through a 0.45 µm filter (20 mm Whatman)), followed by mixing by vortex of the LMWG solution with a hyaluronan containing buffer. Subsequently, the gel was quickly (within 10-30 seconds) injected into the empty lens capsule.

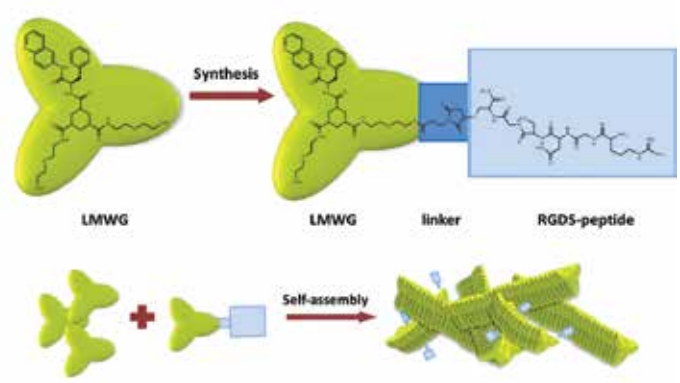


FIGURE 1. *The structure of the LMWG molecule and a schematic representation of the synthesis and nanogel formation. The figure shows the chemical reaction of the coupling of a RGSD peptide to the LMWG molecule by the use of a maleimide linker molecule and the nanogel formation by self-assembly. Self- assembling nanogels are an emerging class of synthetic biomaterials that offer highly bioactive nanostructures and can be of interest for many biomedical applications, as their hydrophilic permeable and flexible characteristics resemble the properties of ECM and thereby provide a (bio) mimetic environment for the LECs as described for other cell types (Cui et al., 2010, Ikonen et al., 2013, Liu et al., 2013).*

Nanogel-peptide combinations

Table 1 provides an overview of the different nanogel-peptide combinations and formulations. The peptide combinations are based on their presence within CO and their origin in the ECM (Walker and Menko, 2009, Wederell and de Iongh, 2006). IKVAV (isoleucine-lysine-valine-alanine-valine) and YIGSR (tyrosine-isoleucine-glycine-serine-arginine) are

both laminin-derived peptides, RGDS (arginine-glycine-aspartic acid-serine) and PHSRN (proline-histidine-serine-arginine-asparagine) are fibronectin-derived, and DGEA (aspartic acid-glycine-glutamic acid-alanine) is a collagen IV-derived peptide.

TABLE 1. *Materials of nanogel-peptide composites*

	Refill material	N	Quantity of peptides
C	Hyaluronan	4	-
1	Nanogel alone	4	-
2	YIGSR	3	0.5 mol%
3	IKVAV	4	0.5 mol%
4	RGDS	4	0.5 mol%
5	DGEA	4	0.5 mol%
6	IKVAV+YIGSR	4	0.5 mol% each
7	RGDS+PHSRN	4	0.5 mol% each
8	RGDS+IKVAV	4	0.5 mol% each
9	RGDS+YIGSR	4	0.5 mol% each
10	RGDS+IKVAV+YIGSR	4	0.5 mol% each
11	RGDS+DGEA	4	0.5 mol% each
12	YIGSR+DGEA	4	0.5 mol% each
13	IKVAV+YIGSR+RGDS+ PHSRN+DGEA	4	0.5 mol% each
14	IKVAV+YIGSR+RGDS+ PHSRN+DGEA	4	PHSRN & DGEA (0.2 mol%) RGDS (0.4 mol%), YIGSR (0.5 mol%), IKVAV (0.6 mol%)

IKVAV&YIGSR are laminin-derived peptides; RGDS&PHSRN are fibronectin-derived peptides; DGEA is a collagen IV-derived peptide.

Most nanogels consisted of a gel with mixed-in peptides at 0.5 mole percent (mol%) each. One group consisted of five peptides of which two peptides (DGEA and PHSRN) were mixed-in at 0.2 mol%, one peptide (RGDS) at 0.4 mol%, one peptide (YIGSR) at 0.5 mol%, and one peptide (IKVAV) at 0.6 mol%. The ratio's of peptides in this latter mix are based on the composition of the basement membrane (Hughes et al., 2010, Kleinman and Martin, 2005).

Surgical refilling procedure

The surgical lens refilling procedure is based on the method previously described by Koopmans et al. (2003, 2006, 2014) and Nibourg et al. (2015a). In short, the lens contents were aspirated through a capsulorhexis of 1.0-1.5 mm diameter. Intentionally no efforts

were made to remove the lens epithelial cell layer. Then, a 3.0 mm diameter capsular plug was inserted to close the capsular bag, and the nanogel was injected until the capsular bag was completely filled and it started to leak around the plug.

Culturing method

The lenses were extracted from the eye and were placed a in culture medium consisting of minimal essential medium (MEM) supplemented with 12% fetal bovine serum (FBS), 2 mM GlutaMAX™-I, 1 mM sodium pyruvate, and 500 Units/ml penicillin – 500 µg/ml streptomycin – 1.25 µg/ml amphotericin B (all Life Technologies Ltd, Paisley, UK). The lenses were cultured for three weeks in a 5% CO2 incubator at 37 °C. The medium was changed twice a week.

Imaging of lens epithelial cells and immunofluorescence

After three weeks of culturing, the lenses were fixated with 3.7% paraformaldehyde for 30 minutes. After permeabilization in 0.5% Triton X-100 in 10 mM phosphate-buffered saline (PBS) for 15 minutes, the LECs were stained for 30 minutes with 2 µg/ml TRITC-labeled phalloidin and 4 µg/ml 4',6-diamidino-2-phenylindole (DAPI) dissolved in PBS containing 1% bovine serum albumin (PBSA). At this stage staining involving antibodies could not be performed as the lens capsular bag was kept intact and the capsule is not permeable for large proteins. Microscopic observation was carried out within one week after fixation with a Leica TCS SP2 Confocal Laser Scanning Microscope (Leica Microsystems, Rijswijk, the Netherlands) for imaging.

Immunofluorescence staining of alpha smooth muscle actin (αSMA), a marker for myofibroblasts, was performed by removing the nanogel from the lenses (Marcantonio et al., 2003). First the plug was removed and the capsule was emptied, therewith allowing free diffusion of antibodies. Subsequently, the capsules were successively incubated in: 5% bovine serum albumin (BSA) in PBS for 30 minutes and a 1:100 dilution of primary antibody mouse-anti-human αSMA (Sigma-Aldrich Co, St. Louis, USA) in PBSA for 1 hour. Next, lenses were washed three times 10 minutes in PBSA and incubated for 1 hour in the secondary antibody, 1:100 diluted FITC-labeled goat-anti-mouse IgG (Jackson ImmunoResearch Europe Ltd, Suffolk, UK) in PBSA. Finally, lenses were washed two times for 5 minutes in PBSA and stored in PBSA until microscopic observation. For microscopic observation intact lens capsular bags were mounted on object slides, covered with citifluor (Citifluor Ltd, London, UK) and cover-slipped.

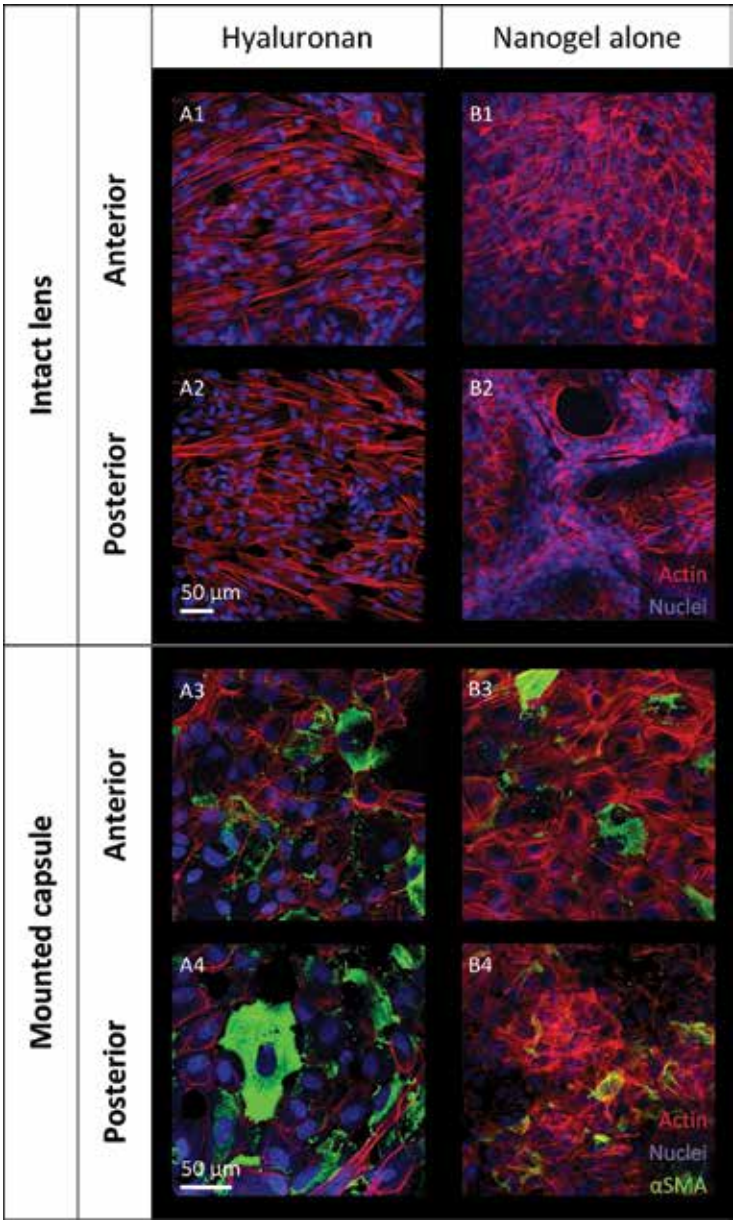


FIGURE 2. *Microscopic images showing the differences in LEC response on the anterior and posterior capsules from lenses filled with hyaluronan (A) and nanogel without peptides (B). Images 1 and 2 are made with the intact lens and show the LEC morphology. Images 3 and 4 are made after immunofluorescence staining of the lens capsules and show the αSMA expression of the LEC on the mounted capsules.*

TABLE 2. *LEC morphology on the confocal images of the anterior and posterior capsules*

	Refill material	Imaging anterior capsule		Imaging posterior capsule			
		transdif.	empty spots	migration	transdif.	wrinkling	clustering
C	Hyaluronan	+		+	+	+	
1	Nanogel alone	+		+	+	+	
2	YIGSR			+			+
3	IKVAV		+	+			+
4	RGDS			+	+	+	
5	DGEA			+	+	+	
6	IKVAV+YIGSR		+	±			
7	RGDS+PHSRN			+	+		
8	RGDS+IKVAV			+	+		
9	RGDS+YIGSR			+	+		
10	RGDS+IKVAV+ YIGSR			+	+	+	
11	RGDS+DGEA			+			
12	YIGSR+DGEA			+	+		
13	IKVAV+YIGSR+ RGDS+PHSRN+ DGEA			+	+	+	
14	IKVAV+YIGSR+ RGDS+PHSRN+ DGEA			+	+	+	

IKVAV&YIGSR are laminin-derived peptides; RGDS&PHSRN are fibronectin-derived peptides; DGEA is a collagen IV-derived peptide; transdif.= transdifferentiation of lens epithelial cells.

Qualification and quantification of the cell response

From each filled lens six images were recorded yielding three images from the anterior and three images from the posterior capsule. First the cell morphology was graded on the images from the intact lens (Table 2). The images from the anterior capsule were graded for the presence of transdifferentiation and empty spots, i.e. parts of the inner capsular wall without the presence of lens epithelial cells. Transdifferentiation was graded when lenses showed stretched cells (Figure 2A1) or an irregular LEC layer (Figure 2B1). In two nanogel-peptide groups we identified empty spots, implying apoptosis of single LECs on the anterior LEC layer (Figure 3C1) or the inability of the LEC to regenerate after damage to the anterior LEC layer due to the surgical procedure (Figure 3F1). The images from the posterior capsule were graded for migration, transdifferentiation, wrinkling, and clustering. Lenses were graded for migration when LECs were situated on the

posterior capsule, because in the natural lens there are no LEC present on the posterior capsule (Figures 2A2 and 2B2). In some nanogel refilled lenses, we noticed wrinkling of the posterior capsule due to contraction of transdifferentiated LECs (e.g. Figure 3A2). Clustering was graded for the presence of clusters or separated sheets of LEC (e.g. Figure 3C2). We did not observe wrinkling or clustering on the anterior capsule.

Secondly, from the images of the mounted capsule the average cell density and the percentage of αSMA positive cells were determined (Table 3) using ImageJ (Schneider et al., 2012). All images showed a monolayer of LEC. Only cells showing a complete cytoplasmatic αSMA staining were counted, including both diffuse staining (e.g. arrow Figure 4B1) and cells possessing αSMA-positive actin filaments (e.g. arrow Figure 4B2).

TABLE 3. *Cell density and percentage of cells with αSMA staining*

	Refill material	Cell density anterior†	Cell density posterior†	% αSMA (± SD)
C	Hyaluronan	522 (724)*		30.08 (24.60)
1	Nanogel alone	36 (14)	232 (58)	9.54 (11.29)
2	YIGSR	134 (41)	208 (41)	2.31 (2.79)
3	IKVAV	132 (62)	196 (51)	2.32 (2.72)
4	RGDS	107 (35)	171 (57)	0.59 (1.18)
5	DGEA	193 (39)	233 (78)	0.35 (0.67)
6	IKVAV+YIGSR	75 (55)	117 (81)	4.62 (6.40)
7	RGDS+PHSRN	68 (32)	191 (96)	6.55 (5.94)
8	RGDS+IKVAV	114 (34)	159 (46)	2.45 (2.75)
9	RGDS+YIGSR	122 (55)	240 (141)	0.72 (1.62)
10	RGDS+IKVAV+YIGSR	124 (35)	165 (46)	1.41 (1.74)
11	RGDS+DGEA	82 (26)	208 (61)	2.46 (4.21)
12	YIGSR+DGEA	160 (46)	200 (62)	6.42 (5.67)
13	IKVAV+YIGSR+RGDS+PHSRN+DGEA	101 (45)	169 (35)	2.15 (1.88)
14	IKVAV+YIGSR+RGDS+PHSRN+DGEA	162 (51)	192 (58)	2.19 (2.20)

IKVAV&YIGSR are laminin-derived peptides; RGDS&PHSRN are fibronectin-derived peptides; DGEA is a collagen IV-derived peptide; αSMA= alpha-smooth muscle actin. Cell density from the anterior capsule of a natural porcine lenses is 244 ± 23.
*For the hyaluronan group we have not distinguished between anterior and posterior images for the cell density.
†Cell density within a field of view of 238 x 238 µm.

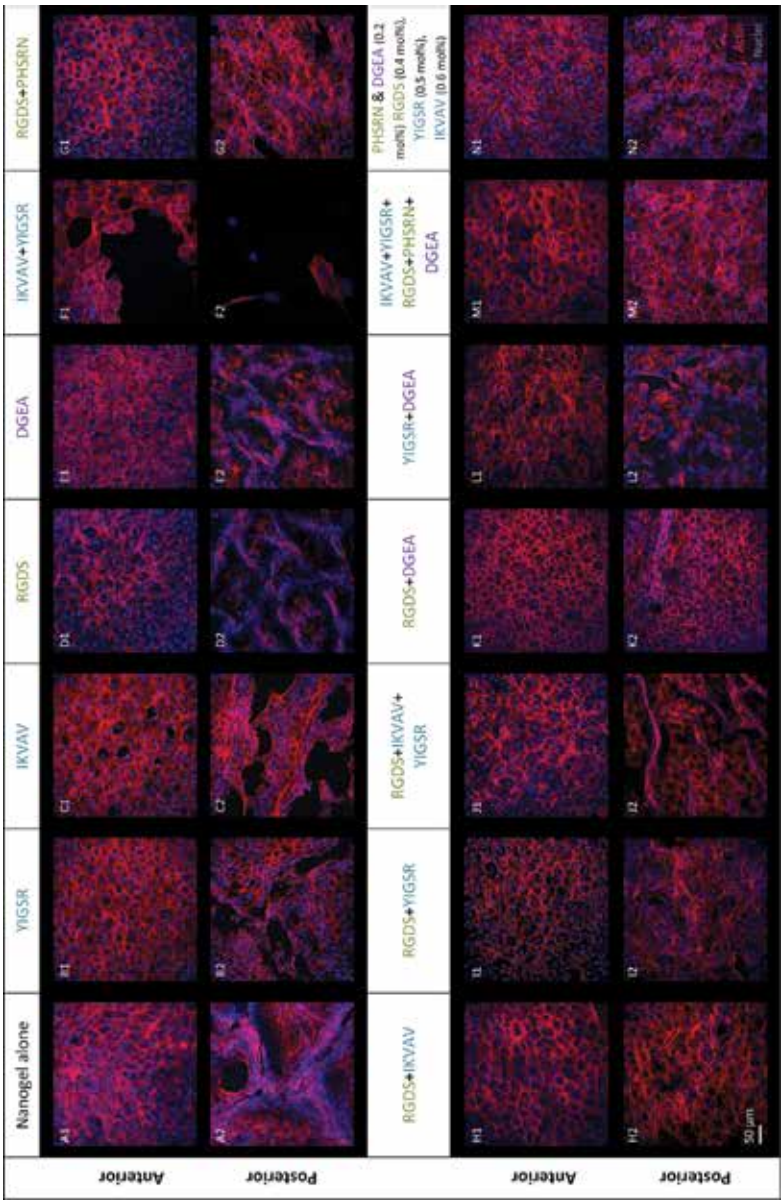


FIGURE 3. *Microscopic images showing the LEC morphology on the anterior and posterior capsules from lenses of the 14 different nanogel-peptide composites. The images from the anterior capsules are numbered with 3A1-3N1; images from the posterior are indicated with 3A2-3N2. It can be seen that lenses form different nanogel-peptide composites show a variety of LEC morphology, ranging from migration to the posterior capsule in combination with transdifferentiation (e.g. image 3A2) to empty spots on the anterior capsule (image 3F1).*

Statistical analyses

The Statistical Package for the Social Sciences (IBM SPSS Statistics 20) was used to analyze the data. Differences between groups in cell density and αSMA staining were evaluated using Wilcoxon rank-sum tests and analyses of variance (ANOVA) with Games-Howell post-hoc analyses. Furthermore, correlations between αSMA expression and cell density on the anterior and posterior capsule were calculated using Pearson's correlation coefficient. The correlation between capsular wrinkling and αSMA expression was calculated using the point-biserial correlation coefficient, since we graded the presence of capsular wrinkling as a dichotomous variable. P values ≤ 0.05 were considered statistically significant.

6.3 Results

CO formation in lenses refilled with hyaluronan compared to nanogel

On both the anterior and posterior capsule many stretched cells containing actin stress fibers were observed in the lenses refilled with hyaluronan, indicating migration of LECs to the posterior capsule and transformation of epithelial cells to myofibroblasts and migration of LEC to the posterior capsule (Figures 2A1 and 2A2). The LECs in the lenses refilled with nanogels showed a more normal epithelial-like morphology, with round shaped cells mixed with cells containing some actin fibers and a few stretched, fully transformed cells (Figures 2B1 and 2B2). Nevertheless, both treatment groups gave a similar score in the qualitative grading (Table 3). However, the expression of αSMA was 30.08±24.60% for the hyaluronan group compared to 9.54±11.29% for the nanogel group (P=0.004, Table 3). This corresponds with the presence of more actin fibers and transdifferentiated cells in lenses refilled with hyaluronan (Figures 2A3 and 2A4) compared to the lenses refilled with nanogel (Figures 2B3 and 2B4).

Formation of CO in lenses refilled with nanogel and nanogel-peptide combinations

Lenses that were filled with nanogel-peptide combinations did not show actin stress fibers and stretched cells on the anterior capsules (Figures 3B1-N1). On the posterior capsules of these lenses, a wide range of cell responses were observed (Figures 3B2-N2), ranging from a few cells on the posterior capsule (Figure 3F2) to capsular wrinkling (Figures 3D2, 3E2, and 3J2) and the formation of actin stress fibers (Figures 3G2, 3H2, 3I2, 3M2, and 3N2). Based on the absence of stress fibers and transformed LEC on the anterior capsules

in the nanogel-peptide refilled groups, CO formation appears less severe in these lenses compared to lenses filled with nanogel alone (Figures 3A1-A2).

Of the lenses filled with nanogels, αSMA expression was highest in lenses refilled with nanogel alone (9.54±11.29%, Table 3), and this was significantly different from the RGDS (0.59±1.18%, P=0.037), DGEA (0.35±0.67%, P=0.029), and RGDS+YIGSR (0.72±1.62%, P=0.043) groups. LEC transformation was also indirectly quantified by assessment of the cell density (Table 2). Transformed LECs increase in size, so a lower cell density in an intact, confluent cell monolayer corresponds with more transformed LECs. There was a correlation between the cell density and the expression of αSMA ($r=-0.381$, $P<0.001$), indicating that a lower cell density, i.e. a larger cell area, corresponds with more transformation. Comparison of all anterior capsules revealed that the lenses refilled with nanogel alone had the lowest cell density (36±14, Table 2/3), and this was different from almost all groups (P values ≤0.013) except for RGDS+PHSRN (68±32, P=0.167) and IKVAV+YIGSR (75±55, P=0.530). These outcomes confirm our observations that CO formation was reduced in lenses refilled with nanogel-peptide combinations compared to lenses refilled with nanogel alone.

LEC response between the different peptide combinations

The anterior capsules of lenses filled with nanogel-peptide combinations did not show LEC transdifferentiation with actin stress fiber formation and stretched cells (Figures 3B1-N1), however, in all groups cell numbers on the anterior capsules were lower compared to the posterior capsules ($P<0.001$, Table 3). Since we observed monolayers of LECs in all lenses, this suggests the presence of larger transformed LECs on the anterior capsules. The response of LECs on the posterior capsules showed a large variation. Only with the IKVAV+YIGSR combination some individual LECs were seen on the posterior capsule (Figure 3F2). Filling of lenses with nanogel-peptide combinations with the laminin-derived peptides IKVAV or YIGSR resulted in sheets of LECs (Figures 3B2 and 3C2). Furthermore, many peptide combinations could not prevent transdifferentiation of LECs with (Figures 3D2, 3E2, and 3J2) or without (Figures 3G2, 3H2, 3I2, 3M2, and 3N2) wrinkling of the posterior capsule. In the lenses filled with the RGDS+DGEA combination, parts of the posterior capsule contained migrated LECs that did not show signs of transdifferentiation (Figure 3K2), however, some parts of the posterior capsules from these lenses filled with RGDS+DGEA were moderately wrinkled. The lenses with the highest αSMA expression were present in the RGDS+DGEA and YIGSR+DGEA groups (Table 3), P values ≤0.026

compared to the lenses in the groups filled with nanogels containing RGDS, DGEA, RGDS+YIGSR, and RGDS+IKVAV+YIGSR. There was no correlation between the presence of capsular wrinkling and αSMA expression (r_{pb}=-0.076, P=0.169).

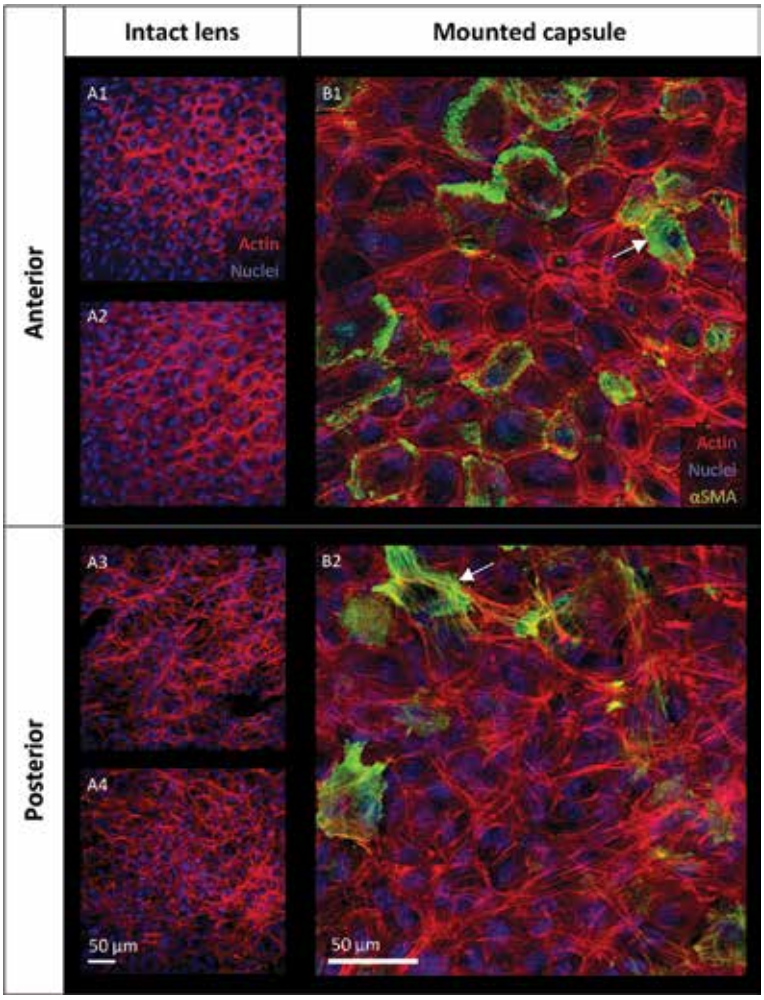


FIGURE 4. *Example of αSMA expression on microscopic images from lenses refilled with the RGDS and PHSRN nanogel-peptide combination. The posterior capsule of these lenses is showing an irregular LEC layer and some stretched cells (4A3-4A4). From the images of the mounted capsule the nuclei were counted to extract the total number of cells and the αSMA expression was assessed (4B1-4B2). Both types of αSMA expression can be seen, including diffuse staining (arrow in 4B1) and cells possessing αSMA-positive actin filaments (arrow in 4B2).*

6.4 Discussion

In this study the response of lens epithelial cells exposed to nanofiber gels with incorporated extracellular matrix-derrived peptides in an *ex vivo* pig eye lens culture model was studied. We show that the LEC response can be influenced by the presence of nanogels with covalently bound cell adhesion mediating peptides that target integrins. LEC transformation was reduced by nanogels compared to hyaluronan control samples and reduced even further in the presence of peptide-nanogels. The cell responses within groups showed variation (e.g. SDs in Table 3). This could firstly be caused by differences in the initial number of anterior LEC after the lens fiber removal as fiber removal can be associated with cell detachment (Nibourg LM, et al. 2015;unpublished data). Variation can also be explained by individual genomic variation of the porcine eyes, since we obtained eyes (n=59) from pigs (n=30-40) at the slaughterhouse. Next to this, we observed a large variation in LEC responses between the 14 different nanogel-peptide combinations (Table 3), showing that the different peptide combinations initialize different cell signaling pathways involved in integrin signaling as well as associated growth factor-related pathways (Mamuya et al., 2014, Wederell and de Iongh, 2006). Integrins can bind a large variety of ligands on ECM components and other molecules involved in cellular signaling (Walker and Menko, 2009, Wederell and de Iongh, 2006). The peptides used in this study are cell-binding domains of the ECM components laminin, fibronectin, and collagen IV. These three ECM components have been described to promote adhesion and migration in a human LEC line and in LEC from rabbit lenses *in vitro*. Also, deposits of these components were found on intraocular lenses from human autopsy samples (Linnola et al., 2000, Olivero and Furcht, 1993). In our study, where a close to natural basement membrane environment was provided, containing laminin, fibronectin, and collagen type-IV derived peptides, we did not observe a reduction in transformation of LECs. This can be due to the fact that the nanogels were provided to the apical side of the cells, which normally does not contain integrin receptors. Therefore, more interesting are the results of the applied nanogels on movement towards and proliferation of LEC on the posterior part of the capsule.

Laminin is normally present in the lens within the basement membrane of the lens epithelium (Parmigiani and McAvoy, 1991). The combination of the laminin-derived peptides IKVAV and YIGSR in our study was the only one which resulted in an absence of cells on parts of the posterior capsule, indicating 'partial' blockage of LEC proliferation

and migration through the interaction with cues from their natural environment. However, cell numbers on the anterior capsule in those lenses were low and αSMA expression was present, both of which are indicators for LEC transdifferentiation. Low cell numbers on the anterior capsule can be the result of the damage to the anterior LEC layer due to the surgery. We did not used any surgical technique aimed at removing anterior LECs and we observed variable damage to the LEC layer after removal of the lens fibers. This wounding of the LEC may have initiated the anterior LEC to transdifferentiate. The fact that these cells did not migrate to the posterior capsule indicates the potential of laminin sequences to maintain the normal LEC morphology or prevent (limit) migration.

Fibronectin is not present in the normal adult lens. Its presence has been associated with abnormal signaling in CO (Mamuya et al., 2014, Oharazawa et al., 1999, Olivero and Furcht, 1993, Parmigiani and McAvoy, 1991). The fact that nanogels containing fibronectin-derived peptides (RGDS and PHSRN) resulted in low cell densities similar to the nanogel without peptides is suggestive for LEC transdifferentiation and indicates that nanogels with combinations with fibronectin-derived peptides are not beneficial in CO prevention. A study by Nishi et al. (1997b) using the RGD peptide for CO prevention showed some inhibition of LEC migration in rabbit eyes by sustained-release from a polylactic acid disk, however, this inhibition was not significant. Our results in lenses that had been filled with the RGDS peptide were also not conclusive. A possible explanation for these results may be that αSMA is not a proper marker for EMT in LEC in our model. In a study on hepatic stellate cells it was found that αSMA expression was independent of the induction of ECM synthesis, which could indicate that both are a result of different signaling pathways (Lindert et al., 2005). We delivered peptides derived from ECM components to the LEC, which may induce EMT through different signaling pathways than the pathways resulting in αSMA expression. In a study focusing on the disintegrin salmosin, it was found that LEC migration and proliferation could be inhibited by targeting integrins (Kim et al., 2002). Disintegrins can interfere with the β-subunit of integrins and salmosin contains different recognition sites for integrins, including those that can be recognized by the RGD peptide. These findings demonstrate that blockage of the RGD recognition sites could also be a target for CO prevention. In other studies involving integrin antagonists inhibition of CO formation by blockage of recognition sites was also reported (Inan et al., 2001, Kim et al., 2002).

Integrin linked kinase (ILK) is involved in integrin signaling after binding of a ligand to the β-subunit of the integrin and to a growth factor receptor (Wederell and de Iongh, 2006). In studies on the developing lens, it was found that blockage of ILK resulted in a

retention of type IV collagen in the ECM, causing irregularities in the lens capsule and illustrating the importance of type IV collagen for LEC behavior (Cammas et al., 2012, Teo et al., 2014). Type IV collagen has been thought to have a protective function for the LEC. It was found that LEC plated on a type IV collagen coating were protected from Fas-dependent apoptosis (Futter et al., 2005). The peptide DGEA in our study is derived from type IV collagen. While DGEA alone did not result in normally shaped LEC (Figure 4E2), the combination RGDS+DGEA incorporated in the nanogel showed LEC with normal morphology (Figure 4K2) and no signs of transformation (Table 2). The reason may be that cells need the initial binding to RGDS before being able to properly interact with DGEA.

Hyaluronan is widely used in the human eye as a component of the viscoelastic solutions that are injected during cataract surgery. For this reason we selected hyaluronan refilled lenses were chosen as a control group. Interestingly, the CD44/hyaluronan pathway has been associated with TGF-β signaling, and both CD44 and hyaluronan were found in human anterior capsules obtained at cataract surgery (Acharya et al., 2008, Nishi et al., 1997a, Saika et al., 1998). Canine LECs treated with different concentrations of hyaluronan showed a dose-dependent increase in expression of CD44 and increased migration of LECs (Chandler et al., 2012). These results match those found in our study and explain the presence of CO in the lenses refilled with hyaluronan. The differences in the formation of CO between lenses refilled with hyaluronan or nanogel-peptide indicate that targeting of integrin signaling using nanogel-peptide indeed provides less EMT. Thus, we provide proof-of-concept for the use of peptide-nanogels to reduce CO formation. By modifying the composition or ratio of peptides or introducing additional ECM-derived peptides optimal nanogel-peptide combinations may be found that fully suppress CO formation.

The model of complete, refilled lenses taken into culture for a period of three weeks proved to be useful for assessing the LEC response. In this study we used medium containing FBS without heat inactivation. Serum contains latent TGF-β as well as other growth factors (Oida and Weiner, 2010). The effects of these factors were not assessed in this study, but could be studied by using defined media. The use of a complete medium containing serum proteins can be regarded as a worst case scenario driving the lens epithelium to transformation. However, it is unclear which of the serum proteins is able to diffuse thought the capsular wall to exert effects. It has been shown that the LECs themselves produce TGF-β that starts their transformation (Meacock et al., 2000, Nishi et al., 1999). This may indicate that the extra-capsular environment is not very relevant to the outcome of the three week culture period.

6.5 Conclusion

Our results show that the LEC response can be influenced by the presence of nanogels and further improved by the incorporation of peptides mediating cell adhesion that target integrins. Interference with integrin signaling targets more than one signaling pathway, which provides a more holistic approach to control LEC behavior for CO prevention. Thus, peptide containing nanomaterials targeting biological pathways are promising tools for CO prevention.

Acknowledgements

The authors thank AMO Groningen for providing the plugs used during the surgery and AMO Netherlands for providing the hyaluronan for this study. The authors would like to express a special word of gratitude to Hepke Gjaltema for optimizing the sterility of the operating environment and to Simone Nibourg for assistance with data processing. This work was supported by the European Fund for Regional Development of the Ministry of Economic Affairs of the Netherlands, Samenwerkingsverband Noord-Nederland (grant number 249-Bioactive interface layers for medical implants), and NanoNextNL, a micro and nanotechnology consortium of the Government of the Netherlands and 130 partners.

References

Acharya P.S., Majumdar S., Jacob M., Hayden J., Mrass P., Weninger W., Assoian R.K., Pure E., 2008. Fibroblast migration is mediated by CD44-dependent TGF beta activation. J. Cell. Sci. 121, 1393-1402.

Brizard A., Stuart M., van Bommel K., Friggeri A., de Jong M., van Esch J., 2008. Preparation of Nanostructures by Orthogonal Self-Assembly of Hydrogelators and Surfactants. Angewandte Chemie International Edition 47, 2063-2066.

Cammas L., Wolfe J., Choi S.Y., Dedhar S., Beggs H.E., 2012. Integrin-linked kinase deletion in the developing lens leads to capsule rupture, impaired fiber migration and non-apoptotic epithelial cell death. Invest. Ophthalmol. Vis. Sci. 53, 3067-3081.

Chandler H.L., Haeussler D.J.,Jr, Gemensky-Metzler A.J., Wilkie D.A., Lutz E.A., 2012. Induction of posterior capsule opacification by hyaluronic acid in an ex vivo model. Invest. Ophthalmol. Vis. Sci. 53, 1835-1845.

Cui H., Webber M.J., Stupp S.I., 2010. Self-assembly of peptide amphiphiles: from molecules to nanostructures to biomaterials. Biopolymers 94, 1-18.

Dawes L.J., Elliott R.M., Reddan J.R., Wormstone Y.M., Wormstone I.M., 2007. Oligonucleotide microarray analysis of human lens epithelial cells: TGFbeta regulated gene expression. Mol. Vis. 13, 1181-1197.

de Iongh R.U., Wederell E., Lovicu F.J., McAvoy J.W., 2005. Transforming growth factor-beta-induced epithelial-mesenchymal transition in the lens: a model for cataract formation. Cells Tissues Organs 179, 43-55.

Futter C.E., Crowston J.G., Allan B.D., 2005. Interaction with collagen IV protects lens epithelial cells from Fas-dependent apoptosis by stimulating the production of soluble survival factors. Invest. Ophthalmol. Vis. Sci. 46, 3256-3262.

Guha R., Chowdhury S., Palui H., Mishra A., Basak S., Mandal T.K., Hazra S., Konar A., 2013. Doxorubicin-loaded MePEG-PCL nanoparticles for prevention of posterior capsular opacification. Nanomedicine (Lond) 8, 1415-1428.

Huang X., Wang Y., Cai J.P., Ma X.Y., Li Y., Cheng J.W., Wei R.L., 2013. Sustained release of 5-fluorouracil from chitosan nanoparticles surface modified intra ocular lens to prevent posterior capsule opacification: an in vitro and in vivo study. J. Ocul. Pharmacol. Ther. 29, 208-215.

Hughes C.S., Postovit L.M., Lajoie G.A., 2010. Matrigel: a complex protein mixture required for optimal growth of cell culture. Proteomics 10, 1886-1890.

Ikonen L., Kerkela E., Metselaar G., Stuart M.C., de Jong M.R., Aalto-Setala K., 2013. 2D and 3D self-assembling nanofiber hydrogels for cardiomyocyte culture. Biomed. Res. Int. 2013, 285678.

Inan U.U., Ozturk F., Kaynak S., Kurt E., Emiroglu L., Ozer E., Ilker S.S., Guler C., 2001. Prevention of posterior capsule opacification by intraoperative single-dose pharmacologic agents. J. Cataract Refract. Surg. 27, 1079-1087.

Kim J.T., Lee D.H., Chung K.H., Kang I.C., Kim D.S., Joo C.K., 2002. Inhibitory effects of salmosin, a disintegrin, on posterior capsular opacification in vitro and in vivo. Exp. Eye Res. 74, 585-594.

Kleinman H.K., Martin G.R., 2005. Matrigel: basement membrane matrix with biological activity. Semin. Cancer Biol. 15, 378-386.

Koopmans S.A., Terwee T., Barkhof J., Haitjema H.J., Kooijman A.C., 2003. Polymer refilling of presbyopic human lenses in vitro restores the ability to undergo accommodative changes. Invest. Ophthalmol. Vis. Sci. 44, 250-257.

Koopmans S.A., Terwee T., Glasser A., Wendt M., Vilupuru A.S., van Kooten T.G., Norrby S., Haitjema H.J., Kooijman A.C., 2006. Accommodative lens refilling in rhesus monkeys. Invest. Ophthalmol. Vis. Sci. 47, 2976-2984.

Koopmans S.A., Terwee T., Hanssen A., Martin H., Langner S., Stachs O., van Kooten T.G., 2014. Prevention of capsule opacification after accommodating lens refilling: pilot study of strategies evaluated in a monkey model. J. Cataract Refract. Surg. 40, 1521-1535.

Lindert S., Wickert L., Sawitza I., Wiercinska E., Gressner A.M., Dooley S., Breitkopf K., 2005. Transdifferentiation-dependent expression of alpha-SMA in hepatic stellate cells does not involve TGF-beta pathways leading to coinduction of collagen type I and thrombospondin-2. Matrix Biol. 24, 198-207.

Linnola R.J., Werner L., Pandey S.K., Escobar-Gomez M., Znoiko S.L., Apple D.J., 2000. Adhesion of fibronectin, vitronectin, laminin, and collagen type IV to intraocular lens materials in pseudophakic human autopsy eyes. Part 1: histological sections. J. Cataract Refract. Surg. 26, 1792-1806.

Liu X., Wang X., Wang X., Ren H., He J., Qiao L., Cui F.Z., 2013. Functionalized self-assembling peptide nanofiber hydrogels mimic stem cell niche to control human adipose stem cell behavior in vitro. Acta Biomater. 9, 6798-6805.

Mamuya F.A., Wang Y., Roop V.H., Scheiblin D.A., Zajac J.C., Duncan M.K., 2014. The roles of alphaV integrins in lens EMT and posterior capsular opacification. J. Cell. Mol. Med. 18, 656-670.

Marcantonio J.M., Syam P.P., Liu C.S., Duncan G., 2003. Epithelial transdifferentiation and cataract in the human lens. Exp. Eye Res. 77, 339-346.

Marcantonio J.M., Vrensen G.F., 1999. Cell biology of posterior capsular opacification. Eye (Lond) 13, 484-488.

McLean S.M., Mathew M.R., Kelly J.B., Murray S.B., Bennett H.G., Webb L.A., Esakowitz L., McLean J.S., 2005. Detection of integrins in human cataract lens epithelial cells and two mammalian lens epithelial cell lines. Br. J. Ophthalmol. 89, 1506-1509.

Meacock W.R., Spalton D.J., Stanford M.R., 2000. Role of cytokines in the pathogenesis of posterior capsule opacification. Br. J. Ophthalmol. 84, 332-336.

Nibourg L.M., Sharma P.K., van Kooten T.G., Koopmans S.A., 2015a. Changes in lens stiffness due to capsular opacification in accommodative lens refilling. Exp. Eye Res. 134, 148-154.

Nibourg L.M., Gelens E., Kuijer R., Hooymans J.M.M., van Kooten T.G., Koopmans S.A., 2015b. Prevention of posterior capsular opacification. Exp. Eye Res. 136, 100-115.

Nishi O., Nishi K., Akaishi T., Shirasawa E., 1997a. Detection of cell adhesion molecules in lens epithelial cells of human cataracts. Invest. Ophthalmol. Vis. Sci. 38, 579-585.

Nishi O., Nishi K., Mano C., Ichihara M., Honda T., Saitoh I., 1997b. Inhibition of migrating lens epithelial cells by blocking the adhesion molecule integrin: a preliminary report. J. Cataract Refract. Surg. 23, 860-865.

Nishi O., Nishi K., Wada K., Ohmoto Y., 1999. Expression of transforming growth factor (TGF)-alpha, TGF-beta(2) and interleukin 8 messenger RNA in postsurgical and cultured lens epithelial cells obtained from patients with senile cataracts. Graefes Arch. Clin. Exp. Ophthalmol. 237, 806-811.

Oharazawa H., Ibaraki N., Lin L.R., Reddy V.N., 1999. The effects of extracellular matrix on cell attachment, proliferation and migration in a human lens epithelial cell line. Exp. Eye Res. 69, 603-610.

Oida T., Weiner H.L., 2010. Depletion of TGF-beta from fetal bovine serum. J. Immunol. Methods 362, 195-198.

Olivero D.K., Furcht L.T., 1993. Type IV collagen, laminin, and fibronectin promote the adhesion and migration of rabbit lens epithelial cells in vitro. Invest. Ophthalmol. Vis. Sci. 34, 2825-2834.

Parmigiani C.M., McAvoy J.W., 1991. The roles of laminin and fibronectin in the development of the lens capsule. Curr. Eye Res. 10, 501-511.

Saika S., Kawashima Y., Miyamoto T., Okada Y., Tanaka S., Yamanaka O., Ohnishi Y., Ooshima A., Yamanaka A., 1998. Immunolocalization of hyaluronan and CD44 in quiescent and proliferating human lens epithelial cells. J. Cataract Refract. Surg. 24, 1266-1270.

Schneider C.A., Rasband W.S., Eliceiri K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671-675.

Teo Z.L., McQueen-Miscamble L., Turner K., Martinez G., Madakashira B., Dedhar S., Robinson M.L., de Iongh R.U., 2014. Integrin linked kinase (ILK) is required for lens epithelial cell survival, proliferation and differentiation. Exp. Eye Res. 121, 130-142.

Tuin A., Gelens E., Bulten H.W., de Jong M.R., 2015. ArtiCYT bio-active self-assembling nanofiber hydrogels: a tunable synthetic ECM alternative to Matrigel for 3D cell culture. *Manuscript in preparation.*

van Bommel K.J., Stuart M.C., Feringa B.L., van Esch J., 2005. Two-stage enzyme mediated drug release from LMWG hydrogels. Org. Biomol. Chem. 3, 2917-2920.

van Kooten T.G., Koopmans S., Terwee T., Norrby S., Hooymans J.M., Busscher H.J., 2006. Development of an accommodating intra-ocular lens--in vitro prevention of re-growth of pig and rabbit lens capsule epithelial cells. Biomaterials 27, 5554-5560.

Walker J., Menko A.S., 2009. Integrins in lens development and disease. Exp. Eye Res. 88, 216-225.

Wang D., Guo D., Bi H., Wu Q., Tian Q., Du Y., 2013. Zinc oxide nanoparticles inhibit Ca-ATPase expression in human lens epithelial cells under UVB irradiation. Toxicol. in. Vitro., 2117-26.

Wederell E.D., de Iongh R.U., 2006. Extracellular matrix and integrin signaling in lens development and cataract. Semin. Cell Dev. Biol. 17, 759-776.

Wormstone I.M., Wang L., Liu C.S., 2009. Posterior capsule opacification. Exp. Eye Res. 88, 257-269.

Worthington J.J., Klementowicz J.E., Travis M.A., 2011. TGFbeta: a sleeping giant awoken by integrins. Trends Biochem Sci 36, 47-54.

Zhang W., Li X., Ye T., Chen F., Sun X., Kong J., Yang X., Pan W., Li S., 2013. Design, characterization, and in vitro cellular inhibition and uptake of optimized genistein-loaded NLC for the prevention of posterior capsular opacification using response surface methodology. Int. J. Pharm. 454, 354-366.

Zhang X.H., Ji J., Zhang H., Tang X., Sun H.M., Yuan J.Q., 2000. Detection of integrins in cataract lens epithelial cells. J. Cataract Refract. Surg. 26, 287-291.

Chapter 7

Effects of peptide ratios in nanofiber-based hydrogels for the prevention of capsular opacification

Lisanne M. Nibourg, Edith Gelens, Simone A.F. Nibourg,
Menno R. de Jong, Roel Kuijer, Theo G. van Kooten,
and Steven A. Koopmans

Abstract

Purpose: An experimental study was performed in which nanofiber-based hydrogels (nanogels) with attached peptides in different ratios were applied to provide an extracellular environment for lens epithelial cells (LECs) in order to modulate the capsular opacification (CO) response after lens surgery in a porcine eye model.

Methods: The lens content was removed and the empty capsules were refilled with nanogel. Lenses were divided into two groups, the first group (n=34) was refilled with different ratios of two laminin-derived peptides (IKVAV+YIGSR) and the latter group (n=26) was refilled with different combinations of a fibronectin-derived and a collagen IV-derived peptide (RGDS+DGEA). Two lenses were refilled with culture medium to investigate the effect of the medium on the LECs. After refilling, lenses were extracted from the porcine eye and cultured for three weeks. LECs were assessed for morphology and alpha smooth muscle actin (αSMA) expression using confocal laser scanning microscopy.

Results: Differences were seen in cell morphology between lenses refilled with nanogels with IKVAV+YIGSR and RGDS+DGEA peptides. In nanogels with IKVAV+YIGSR peptides differences in LEC morphology are largest when ratios between the peptides are not equal, whereas LEC responses from the RGDS+DGEA refilled groups show variation in LEC morphology dependent on the total quantity of mixed-in peptides. Refilling with culture medium did not have an effect on LECs.

Conclusions: Ratios and concentrations of cell adhesion mediating peptides both can direct the LEC response, depending on the adhesion molecules of origin. Nanogels with incorporated peptides probably can be tuned towards CO prevention.

7.1 Introduction

Capsular opacification (CO) following cataract surgery is a common complication leading to a diminished visual acuity as a consequence of proliferation, migration, and transdifferentiation of lens epithelial cells (LECs), a process generally referred to as epithelial to mesenchymal transformation (EMT) (de Iongh et al., 2005, Marcantonio and Vrensen,

1999). In EMT and CO development, there is a key role for TGF-β signaling. However, many other signaling molecules have been reported that influence LEC behavior as well (Nibourg et al., 2015b, Wormstone et al., 2009). Recently, integrin signaling was added to the list of related signaling pathways (Mamuya et al., 2014, Walker and Menko, 2009). Integrin receptors are mainly involved in interactions between cells and extracellular matrix (ECM), and most of the integrin members are present in LECs (McLean et al., 2005, Worthington et al., 2011, Zhang et al., 2000). Following cataract surgery, changes in the extracellular environment of LECs occur. These changes in integrin-ECM interactions are associated with transdifferentiation of LECs, and integrins were found to be activators for TGF-β, which is present in the latent form in the ECM (Dawes et al., 2007, Mamuya et al., 2014, Wederell and de Iongh, 2006, Worthington et al., 2011). Altogether, these processes result in the development of CO.

In a previous study (Nibourg LM, et al. 2015c), we developed an organ-culture porcine eye lens model, in which nanofiber-based hydrogels (nanogels) with attached peptides were applied to interact with the LECs in order to prevent the formation of CO. The lens fibers were removed and nanogels were injected in the remaining capsular bag. This technique is similar to techniques developed for accommodative lens refilling, in which lens fibers are replaced by a silicone polymer enabling accommodation in presbyopic lenses (Koopmans et al., 2003, Koopmans et al., 2006, Koopmans et al., 2014). For the development of these injectable accommodating lenses, CO is a major problem, since these lenses require an intact and clear lens capsule (van Kooten et al., 2006). In our previous study (Nibourg et al. 2015c), promising implications for the use of nanogels for the prevention of CO were demonstrated. The combination of two laminin-derived peptides (IKVAV+YIGSR) showed only a few single LEC on the posterior capsule providing a clear capsule after three weeks of lens culture. Furthermore, the combination of a fibronectin-derived (RGDS) and a collagen IV-derived peptide (DGEA) resulted in a morphologically normal, single LEC layer on the posterior capsule without transformation. Here, we attempt to optimize the nanogel-peptide ratios by using the most promising peptide combinations from the previous study (IKVAV+YIGSR and RGDS+DGEA). The effects of variations in peptide ratios and variation in the model were established using a lens capsule culture model.

7.2 Material and methods

Experimental setup

Sixty-two fresh natural porcine *(Sus domesticus)* eyes were obtained from the local slaughterhouse. All eyes were from pigs with an age of about six months. The lens content was removed, and the lenses were refilled with nanogel (see surgical refilling procedure). The lenses were divided into two groups refilled with nanogel. The first group (n=34) was refilled with different ratios of the laminin-derived peptides IKVAV (isoleucine-lysine-valine-alanine-valine) and YIGSR (tyrosine-isoleucine-glycine-serine-arginine), and the second group (n=26) was refilled with different combinations of the fibronectin-derived peptide RGDS (arginine-glycine-aspartic acid-serine) and the collagen IV-derived peptide DGEA (aspartic acid-glycine-glutamic acid-alanine). There was dropout of one lens from the IKVAV+YIGSR group due to incorrect positioning of the plug causing leaking of the gel from the lens capsule. An additional group of two lenses was refilled with culture medium (see below) to assess the effects of culture medium on the LEC response, e.g. initiation of proliferation or transformation. After extraction from the eye, lenses were kept in minimal essential medium (MEM) supplemented with 12% fetal bovine serum (FBS), 2 mM GlutaMAX™-I, 1 mM sodium pyruvate, and 500 Units/ml penicillin – 500 µg/ml streptomycin – 1.25 µg/ml amphotericin B (all Life Technologies Ltd, Paisley, UK), and cultured for three weeks in a 37 °C 5% CO2 incubator. The culture medium was changed twice a week.

Nanofiber-based hydrogel assembly and preparation

The hydrogel used for refilling of the lenses consists of nanofibers of a low molecular weight hydrogelator (LMWG) and is formed by self-assembly (Figure 1) (Brizard et al., 2008, van Bommel et al., 2004). The synthesis of the hydrogel has previously been described by van Bommel et al. (2005) LMWG was functionalized with a maleimide moiety and subsequently reacted with cysteine-containing peptides to obtain the components for the nanogel-peptide combinations. This process has been described in detail by Tuin et al. (Tuin A, et al. 2015;*manuscript in preparation*). To achieve self-assembly of LMWG into nanofibers and subsequent gel formation, LMWG was first dissolved in a mixture of hydrochloric acid (0.18 M HCl) and saline solution, followed by mixing of the LMWG solution with 5% hyaluronan solution and 3.7% sodium bicarbonate buffer containing 6.5% NaCl by vortex (ratio LMWG:saline:hyaluronan:buffer = 7:30:30:32). Then, the gel

was ready to inject into the empty lens capsule (pH = 6-8). Because of the fast gelation the nanogel had to be injected within 10 seconds. The gelation time slightly varied between gels depending on the amount of peptides which were mixed into the nanogel.

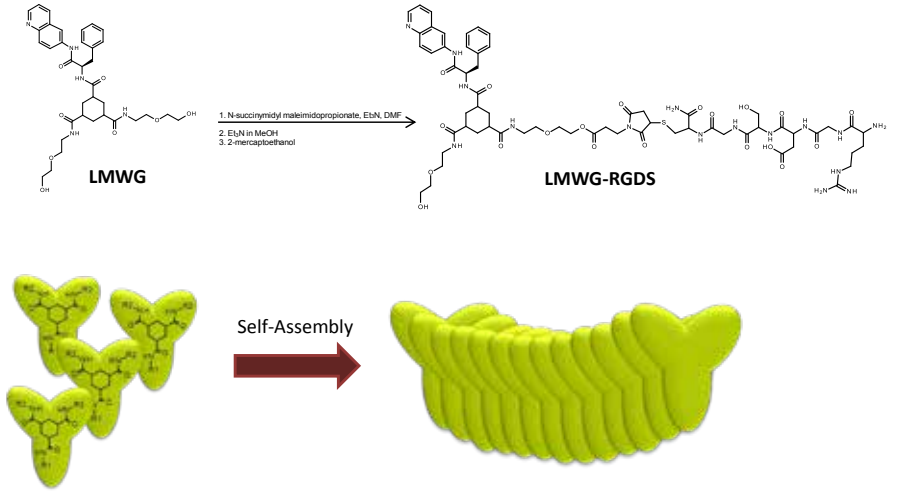


FIGURE 1. *The structure of the LMWG-1 molecule and a schematic representation of the synthesis and nanogel formation. The figure shows the chemical reaction of the coupling of a RGSD peptide to the LMWG-1 molecule by the use of a maleimide linker molecule and the nanogel formation by self-assembly. Self-assembling nanogels are an emerging class of synthetic biomaterials that offer highly bioactive nanostructures and can be of interest for many biomedical applications, as their hydrophilic permeable and flexible characteristics resemble the properties of ECM and thereby provide a (bio) mimetic environment for the LECs as described for other celltypes (Cui et al., 2010, Ikonen et al., 2013, Liu et al., 2013).*

Nanogel-peptide ratios

The peptides derivatives (Nano Fiber Matrices B.V., Groningen, The Netherlands) were mixed into the nanogel in a variety of combinations and ratios (Table 1). In previous experiments, nanogels contained a 0.5 mole percent (mol%) of each peptide derivative. In order to investigate variations to this motif, the quantity of the sum of the peptide derivatives as well as the ratio between them was varied. The laminin-derived peptides IKVAV (isoleucine-lysine-valine-alanine-valine) and YIGSR (tyrosine-isoleucine-glycine-serine-arginine) were mixed in at respectively: 0.25:0.25, 0.5:0.5, 1:1, 1:0.5, 0.5:1, 0.55:0.5, and 0.5:0.55 mol%. The nanogel with the fibronectin-derived RGDS (arginine-

glycine-aspartic acid-serine) and the collagen IV-derived DGEA (aspartic acid-glycine-glutamic acid-alanine) was mixed in the following ratios: 0.25:0.25, 0.5:0.5, 1:1, 1.5:1.5, 1:0.5, and 0.5:1 (mol%).

TABLE 1. *Overview materials and results from the nanogel-peptide refilled lenses.*

	Materials			Results	
	Composition nanogel	N	Quantity of peptides	Cell density (± SD)*	% SMA (± SD)
1	IKVAV + YIGSR	4	0.25 : 0.25 (mol%)	263 (100)	10.39 (9.04)
2	IKVAV + YIGSR	6	0.5 : 0.5 (mol%)	274 (98)	6.87 (10.26)
3	IKVAV + YIGSR	4	1 : 1 (mol%)	222 (55)	6.51 (11.63)
4	IKVAV + YIGSR	3	1 : 0.5 (mol%)	271 (74)	6.85 (5.97)
5	IKVAV + YIGSR	4	0.5 : 1 (mol%)	140 (64)	26.18 (21.92)
6	IKVAV + YIGSR	6	0.55 : 0.5 (mol%)	240 (93)	18.77 (19.31)
7	IKVAV + YIGSR	6	0.5 : 0.55 (mol%)	201 (97)	17.94 (17.64)
8	RGDS + DGEA	4	0.25 : 0.25 (mol%)	167 (86)	5.38 (5.90)
9	RGDS + DGEA	6	0.5 : 0.5 (mol%)	217 (66)	18.30 (12.05)
10	RGDS + DGEA	4	1 : 1 (mol%)	254 (109)	18.20 (19.44)
11	RGDS + DGEA	4	1.5 : 1.5 (mol%)	175 (95)	8.82 (8.87)
12	RGDS + DGEA	4	1 : 0.5 (mol%)	185 (83)	16.34 (11.57)
13	RGDS + DGEA	4	0.5 : 1 (mol%)	161 (83)	15.01 (12.66)

αSMA= alpha-smooth muscle actin. * Cell density within a field of view of 238 x 238 µm.

Surgical refilling procedure

The surgical lens refilling procedure is based on methods described by Koopmans et al. (2003, 2006, 2014) and Nibourg et al. (2015a). To summarize, lens contents were extracted through a capsulorhexis of 1.0-1.5 mm diameter. LECs were not intentionally removed. The capsular bag was closed with a capsular plug (3.0 mm diameter), and the nanogel was injected until complete filling of the capsular bag.

Imaging of lens epithelial cells and immunofluorescence

Lenses were fixated for 30 minutes with 3.7% paraformaldehyde. After permeabilization for 15 minutes in 0.5% Triton X-100 in phosphate-buffered saline (PBS), LECs were stained for 30 minutes with 2 µg/ml tetramethylrhodamine B isothiocyanate (TRITC) labeled phalloidin and 4 µg/ml 4',6-diamidino-2-phenylindole (DAPI) dissolved in PBS containing 1% bovine serum albumin (PBSA).

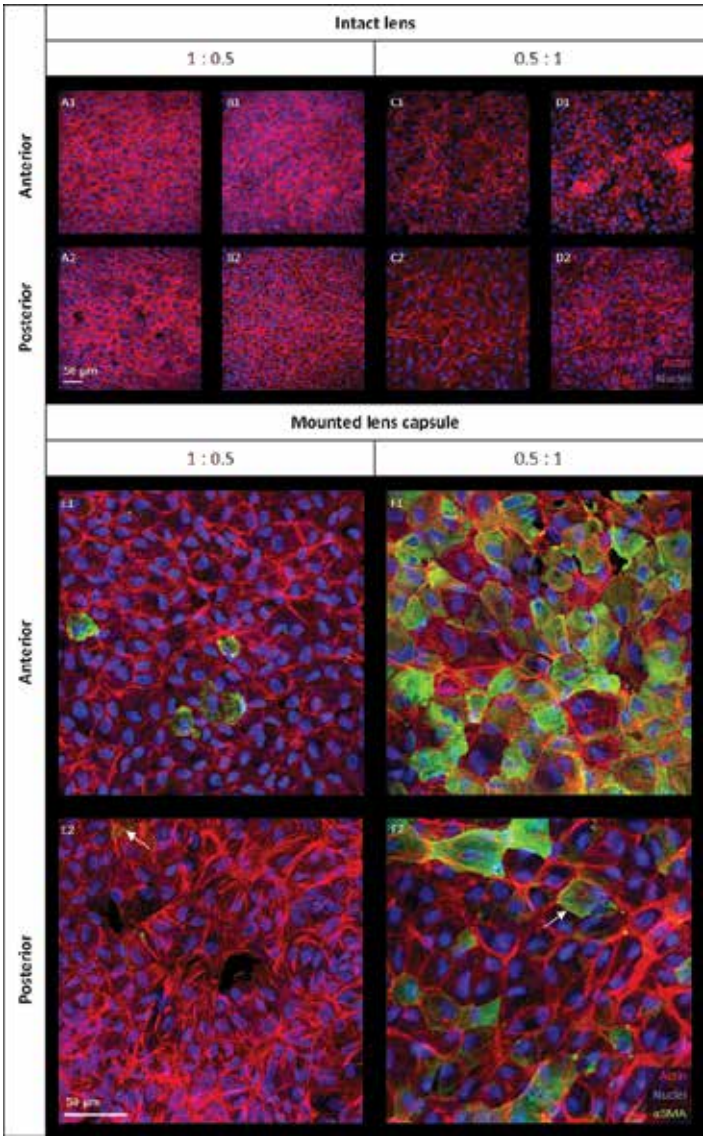


FIGURE 2. *Example of differences in αSMA expression on microscopic images between lenses refilled with respectively 1:0.5 and 0.5:1 mol% IKVAV+YIGSR. First, images from the anterior (2A1-2D1) and posterior capsules (2A2-2D2) of the intact lenses are displayed in order to provide a broader overview of the differences in cell morphology between those groups. Then, images from the mounted anterior (2E1 and 2F1) and posterior capsules (2E2 and 2F2) are shown. From the images of the mounted capsule the nuclei were counted to extract the cell density and the αSMA expression (2E1-2F2). Both types of αSMA expression can be seen, including diffuse staining (arrow in 2F2) and cells possessing αSMA-positive actin filaments (arrow in 2E2). However, αSMA-positive actin filaments were only seen in a few microscopic samples.*

Microscopic observation with the Leica TCS SP2 Confocal Laser Scanning Microscope (Leica Microsystems, Rijswijk, the Netherlands) was carried out on the intact lenses within one week after fixation, until then the lenses were stored in PBS at 4°C in the dark.

The next step was immunofluorescence staining for the myofibroblasts marker alpha smooth muscle actin (αSMA) (Marcantonio et al., 2003). To perform this staining, the nanogel was drawn from the lenses by removing the plug and emptying the capsule. Then, the capsules were incubated for 30 minutes in 5% bovine serum albumin (BSA) in PBS, followed by incubation for 1 hour with a 1:100 dilution of primary mouse-anti-human αSMA antibody (A2547, Sigma-Aldrich Co, St. Louis, USA) in PBSA. Next, lenses were washed three times 10 minutes in PBSA and incubated for 1 hour in the secondary antibody, 1:100 diluted FITC-labeled goat-anti-mouse IgG (Jackson ImmunoResearch Europe Ltd, Suffolk, UK) in PBSA. Lenses were washed two times 5 minutes in PBSA, and were stored in PBSA until microscopic observation. For microscopic observation lens capsular bags were mounted on object slides, covered with citifluor (Citifluor Ltd, London, UK) and cover-slipped.

Qualification and quantification of the cell response

From each lens six images were recorded, yielding three images of the anterior and three images of the posterior capsule. Images of the anterior capsule were taken from the exact middle of the anterior capsule (top), an image from the proximal capsule nearby the plug (plug) and an image away from the plug. To evaluate effects of EMT on our samples, images were analyzed for changes in cell morphology, quantified as size of the cell. The average cell density and the percentage of αSMA positive cells were determined using ImageJ software (Schneider et al., 2012). All images showed a monolayer of LEC, and only cells showing a complete cytoplasmatic αSMA staining were counted as αSMA positive, including both diffuse staining (e.g. arrow Figure 2F2) and cells possessing αSMA-positive actin filaments (e.g. arrow Figure 2E2). The percentage αSMA positive cells was then determined from the total cell count per image.

Assessment of lens epithelial cell layers post-surgery

LEC layers post-surgery were assessed using pictures made with the camera attached to the surgical microscope (OPMI 6-SFC, Carl Zeiss Meditec AG, Oberkochen, Germany) direct after the surgical procedure (Figure 3). Since it was not possible to assess initial LEC layers in lenses refilled with LMWG because these solutions were not transparent (Figure

4), the two lenses in Figure 3 were filled with an optically clear viscoelastic solution to show the anterior LEC layers after removal of the lens contents.

Statistical analyses

The data was analyzed with the Statistical Package for the Social Sciences (IBM SPSS Statistics 20). Differences in cell density and αSMA expression were evaluated using Wilcoxon rank-sum tests and analyses of variance (ANOVA) with Games-Howell post-hoc analyses. In the IKVAV+YIGSR groups, differences in cell density between top and plug images from the anterior capsules were evaluated with the Wilcoxon signed-rank test for paired samples. P values ≤ 0.05 were considered statistically significant.

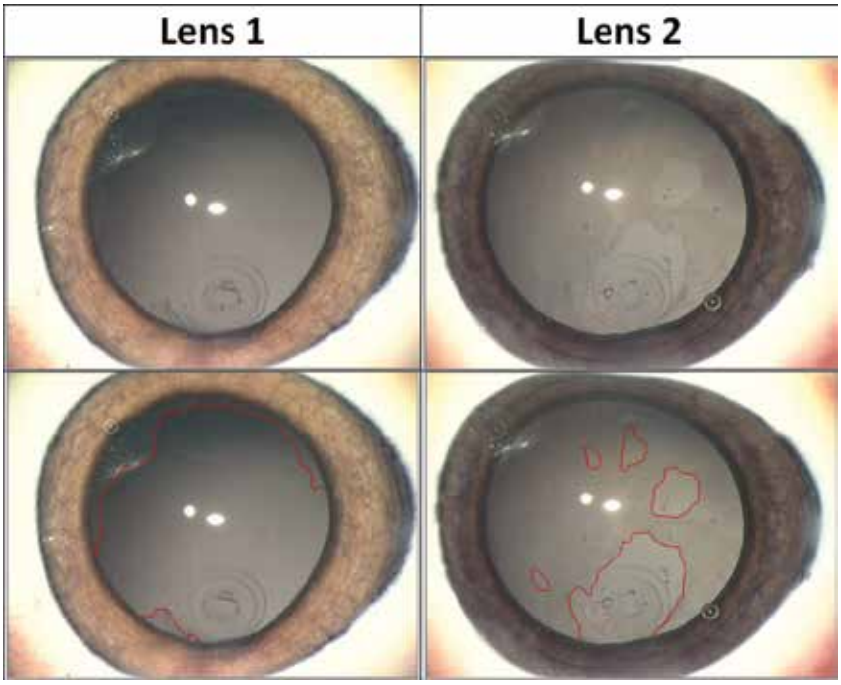


FIGURE 3. *Variation in post-surgical damage of the anterior LEC layer in two different porcine lenses refilled with a viscoelastic solution (sodium hyaluronate, Healon 10 mg/ml; Abbot Medical Optics, Uppsala, Sweden). Pictures are taken direct after the surgical procedure. In the bottom pictures, the borders of the LEC layers are indicated in red. The figure shows that there is variation in initial LEC layers between lenses at the start of the culturing period. The pattern of damage to the LEC layer in lens 2 can be explained by the handling of the cannula during the extraction of the lens fibers.*

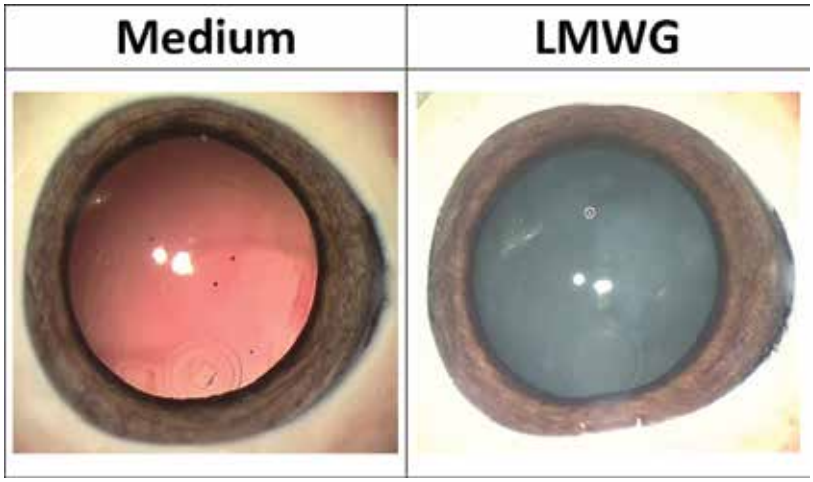


FIGURE 4. *Images of porcine lenses immediately after refilling with LMWG and culture medium*

7.3 Results

Differences in LEC response between IKVAV+YIGSR and RGDS+DGEA groups

The LEC response varied between the two different peptide combinations (Figures 5 and 6). LEC in the IKVAV+YIGSR groups were smaller ($P < 0.001$), but there were no differences in αSMA expression between IKVAV+YIGSR and RGDS+DGEA nanogels (Table 1, $P = 0.112$). In both groups, the microscopic samples showed mainly round regular LEC layers (e.g. Figure 5B1 and 6D1), interspersed with some samples with a slightly more irregular LEC pattern (e.g. Figure 5G1 and 6A2). Strings of bundled LECs in tubular structures were only seen in the RGDS+DGEA groups (e.g. Figure 6E1).

LEC response in the IKVAV+YIGSR nanogel groups

In the groups filled with the laminin-derived peptides IKVAV+YIGSR the differences in LEC morphology were largest when there were large differences between the ratios of both peptides (Figure 5D1-5G2). This also accounted for quantitative measures in these groups, as the variation in peptide ratios from respectively 1:0.5 to 0.5:1 mol% IKVAV+YIGSR in the nanogels (Figure 7, part B) provided significant differences in αSMA expression ($P = 0.007$) and cell density ($P < 0.001$). In samples containing the 0.5:0.55 mol% ratios nanogels, however, a similar trend can be seen in cell densities (Figure 7,

part C). No variation in αSMA expression and cell density was seen in the groups which contained nanogels that differed in total peptide amount with equal ratios (Figure 7, part A, P values ≥ 0.142). Together these results suggest that for the combination of the two laminin-derived peptides IKVAV and YIGSR differences in peptide ratios were more important for cell signaling than differences in total peptide concentration.

LEC response in the RGDS+DGEA nanogel groups

Contrary to the results from the IKVAV+YIGSR groups, responses from the RGDS+DGEA refilled groups showed variation in LEC morphology dependent on the total quantity of mixed-in peptides (Figure 6A1- 6D2). Most notable were the large LECs on the anterior capsule observed in the lens filled with the 0.25:0.25 mol% nanogel (Figure 6A1), and also the LECs in the lens filled with the 1.5:1.5 nanogel appeared larger compared to the 0.5:0.5 and 1:1 lenses. This difference was also reflected in the αSMA expression and cell density, lower in the lenses filled with the 0.25:0.25 and 1.5:1.5 mol% nanogels compared to the lenses with the 0.5:0.5 and 1:1 nanogels (P values ≤ 0.47). The boxplots in Figure 7 part D show a slightly parabolic correlation between the different quantities of peptides in the nanogel. There were no differences in cell morphology (Figure 6E1 and 6E2), αSMA expression (P = 0.998), and cell density (P = 0.919) when ratios between the RGDS and DGEA peptides are varied (Figure 7, part E). Therefore, we chose not to perform refilling experiments with peptide ratios of 0.5:0.55 mol% for this combination. Thus, for the fibronectin-derived RGDS and the collagen IV-derived DGEA the total peptide amount was more important than changes in peptide ratios.

Other factors for variation in LEC response after nanogel refilling

Next to variation in peptide ratios, we also found differences in initial LEC layers post-surgery (Figure 3). In lens 1, LECs were only seen at the periphery of the anterior capsule, while in lens 2 LECs were present over the whole anterior capsule. The pattern of damage to the LEC layer in lens 2 can be explained by the handling of the cannula during the extraction of the lens fibers. At this time, no attempts were made to clear the anterior capsule from LECs. Still the lens extraction procedure created a large variation in initial LEC layers between lenses at the start of the culturing period.

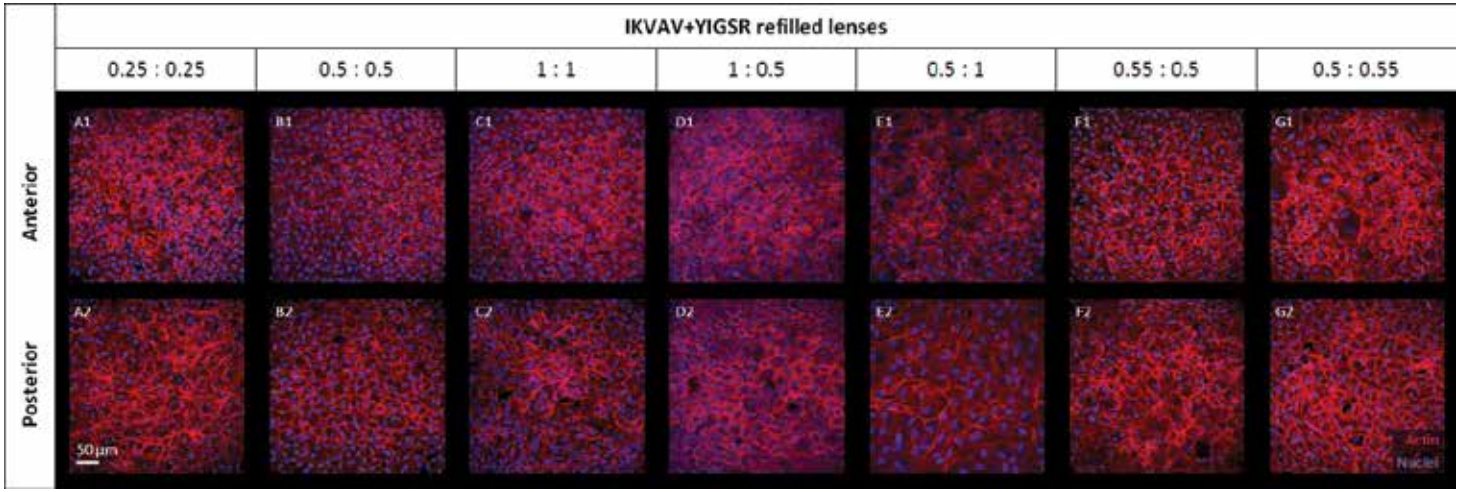


FIGURE 5. *Microscopic images showing the LEC morphology on the anterior and posterior capsules from lenses of the IKVAV+YIGSR refilled groups. The amount of mixed-in peptides in mol% from respectively IKVAV and YIGSR are shown to indicate the groups. Images from the anterior capsules are numbered with 2A1-2G1; images from the posterior are indicated with 2A2-2G2. Differences in cell morphology can be seen between the 1:0.5 and 0.5:1 lenses, in which the LECs from the 0.5:1 lens (5E1 and 5E2) are larger compared to the LECs from the 1:0.5 lens (5D1 and 5D2). Furthermore, LECs from the anterior part of the 0.5:0.55 lens (5G1) show a variety in cell sizes, with larger sized LECs compared to other groups.*

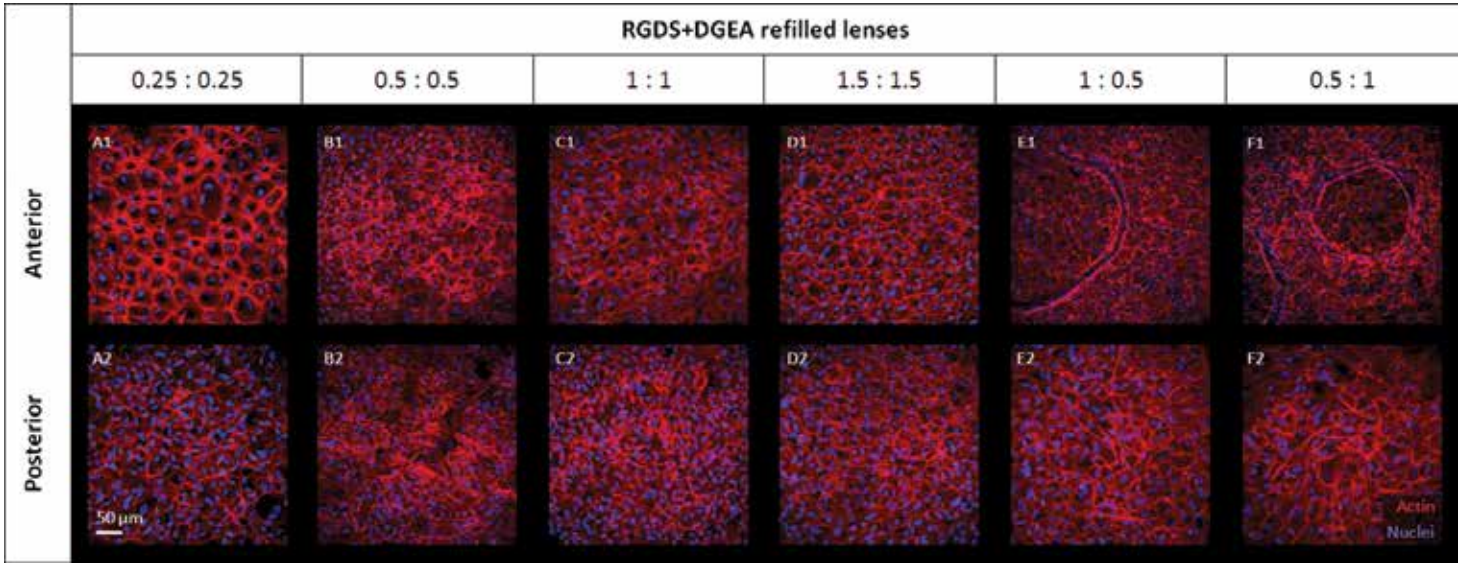


FIGURE 6. *Microscopic images showing the LEC morphology on the anterior and posterior capsules from lenses of the RGDS+DGEA refilled groups. The amount of mixed-in peptides in mol% from respectively RGDS and DGEA are shown to indicate the groups. Images from the anterior capsules are numbered with 6A1-6F1; images from the posterior are indicated with 6A2-6F2. LECs from the anterior capsules of the 0.25:0.25 (6A1) and 1.5:1.5 (6D1) lenses appear larger, whereby the LECs in the 0.25:0.25 clearly are the largest. Furthermore, it is notable that the LECs from the anterior capsules of the 1:0.5 (6E1) and 0.5:1 (6F1) lenses show bundles of LECs growing in strings on top of the existing LEC layer. This was seen in all samples, but not in all parts of the anterior capsules.*

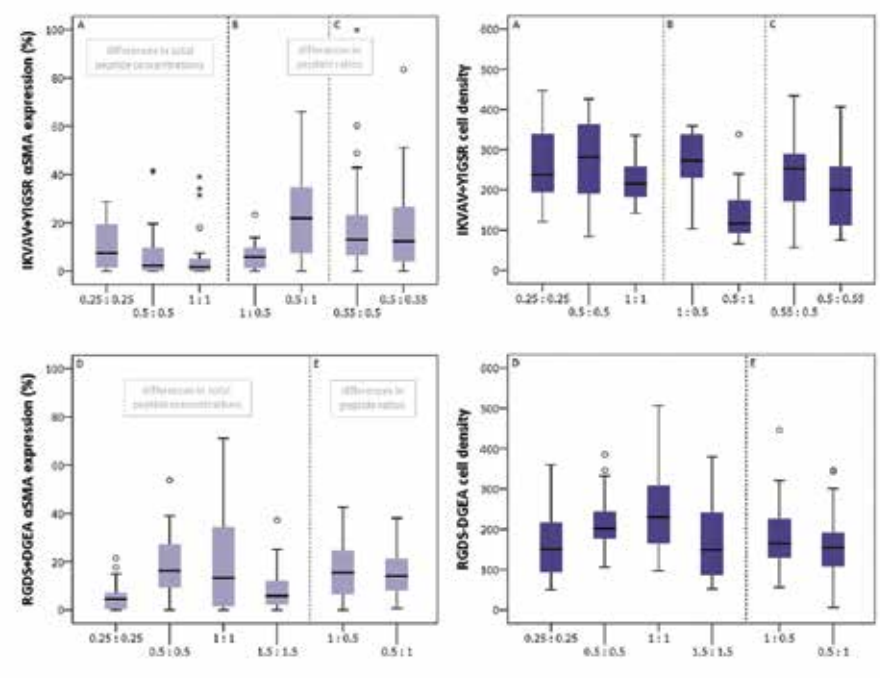


FIGURE 7. *Boxplots of αSMA expression and cell density in the IKVAV+YIGSR and RGDS+DGEA refilled groups. In the plots for the IKVAV+YIGSR groups, first the differences between peptide concentrations are shown (part A) and then the differences in peptide ratios (part B and C). For the RGDS+DGEA groups, differences in total peptide concentrations (part D) and differences in peptide ratios (part E) are shown. It can be seen that differences in peptide ratios are important for the IKVAV+YIGSR groups and that differences in total peptide concentrations causes variation in the RGDS+DGEA groups for both αSMA expression and cell density.*

Assessment of the effects of culture medium on the LEC response showed that the cell density in the medium refilled lenses (236 ± 23 LECs in a field of view of 238 x 238 µm) was comparable to the cell density in natural porcine lenses directly after extraction (244 ± 23 LECs, Nibourg et al. 2015c), indicating that the effect of the growth factors in FBS containing medium on LECs was negligible in our culture model.

7.4 Discussion

The current study shows that the lens epithelium response post cataract surgery can be influenced by adding nanogels with varying ratios or total concentrations of cell adhesion mediating peptides, dependent on the type of peptides. To our knowledge, this is the first study that compares the effect of ratios and concentrations of cell adhesion mediating peptides incorporated in nanogels on lens epithelial cell behavior. Small changes in the quantities of peptides were found to have large effects on cell morphology (size). The findings in this study complement those of an earlier study, in which nanogels with 13 different peptide combinations (including RGDS+DGEA and IKVAV+YIGSR in 0.5:0.5 mol%) were examined in a porcine lens culture system, showing a reduction of LEC transformation by nanogels which was improved by the incorporation of peptides into the nanogels (Nibourg et al. 2015c). The current study does not show severe LEC transformation in the form of cellular spreading (Figures 5 and 6) as seen in the nanogel without peptides from the previous study, so refilling the lens capsule with nanogel-peptide combinations appeared to be a viable method for reducing LEC transformation. Furthermore, the current study elaborates on the impact of integrin signaling of the specific peptide combinations present in the nanogels as discussed below.

While we previously described areas without cell growth with IKVAV:YIGSR 0.5:0.5, we decided to study combinations of these two peptides in more detail for this study, as laminin is present in the normal basal lamina and we have found since the previous study that the total absence of cells in certain regions may well be an artifact of the surgical technique (Parmigiani and McAvoy, 1991). There must have been differences in initial LEC layers post-surgery as we noticed a wide variation in the presence of LECs on the anterior capsule, probably due to handling of the instruments during surgery (Figure 3). Differences in initial LEC numbers may be exerting variation within groups, which may have resulted in larger SD's for the αSMA expression and cell density (Figure 7 and Table 1). The nanogels with incorporated laminin-derived peptides showed a variety of LEC responses dependent on the peptide ratios. Differences in LEC morphology within the laminin-based nanogels were found to be largest when concentrations of the IKVAV and the YIGSR peptide were unequal (Figure 2 and 7), which is an uncommon environment as IKVAV and YIGSR are equally distributed in the basal lamina. It is possible, however, that both cell adhesion-mediating groups may not be available to the cells in equal amounts as they can be cryptic. The relatively small cells with low αSMA expression in e.g. the 0.5:0.5

and 1:0.5 mol% groups (Figure 5B1-5B2 and 5D1-5D2) are suggestive of morphologically normal LECs in these groups.

In the second group, combinations of a fibronectin-derived peptide and a collagen IV-derived peptide were used, since these combinations provided a regular round-shaped LEC-layer in our previous study (Nibourg et al. 2015c). Generally, the presence of fibronectin in the lens has been associated with abnormal LEC signaling (Oharazawa et al., 1999, Olivero and Furcht, 1993, Parmigiani and McAvoy, 1991), and collagen IV has been thought to have a protective function for the LEC (Futter et al., 2005). Our previous study showed that the collagen IV-derived peptide DGEA alone did not maintain LECs with normal morphology, while the combination of DGEA and the fibronectin-derived RGDS did provide normal shaped LECs (Nibourg et al. 2015c). A possible explanation may be that the cells need the initial binding to RGDS before interaction with DGEA is possible. LECs from RGDS+DGEA refilled lenses from the current study show a variety of morphological responses based on the various peptide ratios. Differences in total quantity of mixed-in peptides had the largest effect on LEC morphology between groups (Figures 6 and 7). The slightly parabolic correlation in αSMA expression and cell density between groups in Figure 7 part D reveals that αSMA expression is not always correlated to larger sized LECs, illustrating that in our model αSMA alone may not be the most appropriate marker for LEC transformation. A study on hepatic stellate cells indicated that αSMA expression could result from other signaling pathways than those initiating ECM synthesis (Lindert et al., 2005). We also demonstrated that for maintaining normal LEC morphology the optimal peptide quantity of RGDS+DGEA is around a total of 1-2 mol% (Figure 6B1 and 6C2). Lower and higher amounts of peptides in equal ratios resulted in larger LECs (Figure 6A1 and 6D1), whereas the nanogels with unequal peptide ratios showed remarkable LEC morphology with round shaped strings of cells on top of the LEC layer (Figure 6E1 and 6E2). As far as we know, these tube-like ring-structured LEC strings have not been described before. The LECs in these strings did not show elongation or αSMA expression and form a border with the surrounding LEC sheets. So it is seems more likely that the strings are formed in the epithelial cell layer and are not a result from EMT-associated transformation.

Similarly to our previous study, lens cultures in this study were performed using FBS containing medium. Thus, latent TGF-β as well as other growth factors were available to the LECs (Oida and Weiner, 2010). In the current study, we did not find an effect of culture medium on LEC size and number compared to LECs from natural porcine lenses

(Nibourg et al. 2015c). According to these data we may conclude that the culture medium did not influence the LEC response in this study. Furthermore, it needs to be considered that the use of medium with FBS can be regarded as a worst case scenario in which LEC transformation is induced because of the exposure to growth factors (Nibourg et al. 2015c). Although it is unknown which of the serum proteins can diffuse through the capsular wall and exert effects on LEC transformation, our findings suggest that the extra-capsular environment is less relevant to the outcome and that the used model is effective for the assessment of LEC responses following nanogel refilling.

Our findings enhance the understanding of integrin signaling in LECs by highlighting the importance of ratios and concentrations of cell adhesion mediating peptides in LEC-signaling. It has been shown that LEC responses are directed via integrin signaling (Inan et al., 2001, Nishi et al., 1997). Our data revealed that small changes in peptide quantities have a profound effect on LEC morphology in porcine lens cultures. Thus, the use of nanogels with incorporated peptides can be a promising method towards tuning lens epithelial cells behavior and therewith CO prevention. Further studies on peptide ratios are therefore highly recommended.

Acknowledgements

The authors want to thank AMO Groningen for providing the plugs used during the surgery and AMO Netherlands for providing the sodium hyaluronate for this study. This work was supported by the European Fund for Regional Development of the Ministry of Economic Affairs of the Netherlands, Samenwerkingsverband Noord-Nederland (grant number 249-Bioactive interface layers for medical implants), and NanoNextNL, a micro and nanotechnology consortium of the Government of the Netherlands and 130 partners.

References

Brizard A., Stuart M., van Bommel K., Friggeri A., de Jong M., van Esch J., 2008. Preparation of Nanostructures by Orthogonal Self-Assembly of Hydrogelators and Surfactants. Angewandte Chemie International Edition 47, 2063-2066.

Cui H., Webber M.J., Stupp S.I., 2010. Self-assembly of peptide amphiphiles: from molecules to nanostructures to biomaterials. Biopolymers 94, 1-18.

Dawes L.J., Elliott R.M., Reddan J.R., Wormstone Y.M., Wormstone I.M., 2007. Oligonucleotide microarray analysis of human lens epithelial cells: TGFbeta regulated gene expression. Mol. Vis. 13, 1181-1197.

de Iongh R.U., Wederell E., Lovicu F.J., McAvoy J.W., 2005. Transforming growth factor-beta-induced epithelial-mesenchymal transition in the lens: a model for cataract formation. Cells Tissues Organs 179, 43-55.

Futter C.E., Crowston J.G., Allan B.D., 2005. Interaction with collagen IV protects lens epithelial cells from Fas-dependent apoptosis by stimulating the production of soluble survival factors. Invest. Ophthalmol. Vis. Sci. 46, 3256-3262.

Ikonen L., Kerkela E., Metselaar G., Stuart M.C., de Jong M.R., Aalto-Setala K., 2013. 2D and 3D self-assembling nanofiber hydrogels for cardiomyocyte culture. Biomed. Res. Int. 2013, 285678.

Inan U.U., Ozturk F., Kaynak S., Kurt E., Emiroglu L., Ozer E., Ilker S.S., Guler C., 2001. Prevention of posterior capsule opacification by intraoperative single-dose pharmacologic agents. J. Cataract Refract. Surg. 27, 1079-1087.

Koopmans S.A., Terwee T., Barkhof J., Haitjema H.J., Kooijman A.C., 2003. Polymer refilling of presbyopic human lenses in vitro restores the ability to undergo accommodative changes. Invest. Ophthalmol. Vis. Sci. 44, 250-257.

Koopmans S.A., Terwee T., Glasser A., Wendt M., Vilupuru A.S., van Kooten T.G., Norrby S., Haitjema H.J., Kooijman A.C., 2006. Accommodative lens refilling in rhesus monkeys. Invest. Ophthalmol. Vis. Sci. 47, 2976-2984.

Koopmans S.A., Terwee T., Hanssen A., Martin H., Langner S., Stachs O., van Kooten T.G., 2014. Prevention of capsule opacification after accommodating lens refilling: pilot study of strategies evaluated in a monkey model. J. Cataract Refract. Surg. 40, 1521-1535.

Lindert S., Wickert L., Sawitza I., Wiercinska E., Gressner A.M., Dooley S., Breitkopf K., 2005. Transdifferentiation-dependent expression of alpha-SMA in hepatic stellate cells does not involve TGF-beta pathways leading to coinduction of collagen type I and thrombospondin-2. Matrix Biol. 24, 198-207.

Liu X., Wang X., Wang X., Ren H., He J., Qiao L., Cui F.Z., 2013. Functionalized self-assembling peptide nanofiber hydrogels mimic stem cell niche to control human adipose stem cell behavior in vitro. Acta Biomater. 9, 6798-6805.

Mamuya F.A., Wang Y., Roop V.H., Scheiblin D.A., Zajac J.C., Duncan M.K., 2014. The roles of alphaV integrins in lens EMT and posterior capsular opacification. J. Cell. Mol. Med. 18, 656-670.

Marcantonio J.M., Syam P.P., Liu C.S., Duncan G., 2003. Epithelial transdifferentiation and cataract in the human lens. Exp. Eye Res. 77, 339-346.

Marcantonio J.M., Vrensen G.F., 1999. Cell biology of posterior capsular opacification. Eye (Lond) 13, 484-488.

McLean S.M., Mathew M.R., Kelly J.B., Murray S.B., Bennett H.G., Webb L.A., Esakowitz L., McLean J.S., 2005. Detection of integrins in human cataract lens epithelial cells and two mammalian lens epithelial cell lines. Br. J. Ophthalmol. 89, 1506-1509.

Nibourg L.M., Sharma P.K., van Kooten T.G., Koopmans S.A., 2015a. Changes in lens stiffness due to capsular opacification in accommodative lens refilling. Exp. Eye Res. 134, 148-154.

Nibourg L.M., Gelens E., Kuijer R., Hooymans J.M.M., van Kooten T.G., Koopmans S.A., 2015b. Prevention of posterior capsular opacification. Exp. Eye Res. 136, 100-115.

Nishi O., Nishi K., Mano C., Ichihara M., Honda T., Saitoh I., 1997. Inhibition of migrating lens epithelial cells by blocking the adhesion molecule integrin: a preliminary report. J. Cataract Refract. Surg. 23, 860-865.

Oharazawa H., Ibaraki N., Lin L.R., Reddy V.N., 1999. The effects of extracellular matrix on cell attachment, proliferation and migration in a human lens epithelial cell line. Exp. Eye Res. 69, 603-610.

Oida T., Weiner H.L., 2010. Depletion of TGF-beta from fetal bovine serum. J. Immunol. Methods 362, 195-198.

Olivero D.K., Furcht L.T., 1993. Type IV collagen, laminin, and fibronectin promote the adhesion and migration of rabbit lens epithelial cells in vitro. Invest. Ophthalmol. Vis. Sci. 34, 2825-2834.

Parmigiani C.M., McAvoy J.W., 1991. The roles of laminin and fibronectin in the development of the lens capsule. Curr. Eye Res. 10, 501-511.

Schneider C.A., Rasband W.S., Eliceiri K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671-675.

Tuin A., Gelens E., Bulten H.W., de Jong M.R., 2015. ArtiCYT bio-active self-assembling nanofiber hydrogels: a tunable synthetic ECM alternative to Matrigel for 3D cell culture. *Manuscript in preparation.*

van Bommel K.J., Stuart M.C., Feringa B.L., van Esch J., 2005. Two-stage enzyme mediated drug release from LMWG hydrogels. Org. Biomol. Chem. 3, 2917-2920.

van Bommel K.J., van der Pol C., Muizebelt I., Friggeri A., Heeres A., Meetsma A., Feringa B.L., van Esch J., 2004. Responsive cyclohexane-based low-molecular-weight hydrogelators with modular architecture. Angew. Chem. Int. Ed Engl. 43, 1663-1667.

van Kooten T.G., Koopmans S., Terwee T., Norrby S., Hooymans J.M., Busscher H.J., 2006. Development of an accommodating intra-ocular lens--in vitro prevention of re-growth of pig and rabbit lens capsule epithelial cells. Biomaterials 27, 5554-5560.

Walker J., Menko A.S., 2009. Integrins in lens development and disease. Exp. Eye Res. 88, 216-225.

Wederell E.D., de Iongh R.U., 2006. Extracellular matrix and integrin signaling in lens development and cataract. Semin. Cell Dev. Biol. 17, 759-776.

Wormstone I.M., Wang L., Liu C.S., 2009. Posterior capsule opacification. Exp. Eye Res. 88, 257-269.

Worthington J.J., Klementowicz J.E., Travis M.A., 2011. TGFbeta: a sleeping giant awoken by integrins. Trends Biochem Sci 36, 47-54.

Zhang X.H., Ji J., Zhang H., Tang X., Sun H.M., Yuan J.Q., 2000. Detection of integrins in cataract lens epithelial cells. J. Cataract Refract. Surg. 26, 287-291.

Chapter 8

Preservation of enucleated porcine eyes for use in a wet laboratory

Lisanne M. Nibourg and Steven A. Koopmans

This chapter is an edited version of the manuscript: Nibourg L.M., Koopmans S.A., 2014. Preservation of enucleated porcine eyes for use in a wet laboratory. Journal of Cataract and Refractive Surgery. 40, 644-651. (Re-use permitted)

Abstract

Purpose: To design a method to preserve enucleated porcine eyes for use in a wet laboratory.

Setting: Laboratory of Experimental Ophthalmology, University Medical Center Groningen, the Netherlands.

Design: Experimental study.

Methods: Porcine eyes were preserved using 15 methods including salt solutions, anterior chamber infusion fluids, tap water, mineral water, air, and topical glycerol on the cornea. The central corneal thickness (CCT) was measured by A-scan ultrasound over 3 days. Differences between increases in CCT were compared using repeated-measures multivariate analyses of variance. Also, lenses from eyes preserved in tap water were extracted and the lens epithelial cell morphology was studied.

Results: There was a significant interaction between the preservation method and CCT over 3 days ($P < 0.001$). Post-hoc tests showed that the CCT in the sodium chloride (NaCl) 10% and tap water groups increased significantly less than with four other preservation methods. However, preservation in NaCl 10% resulted in unusable shrunken coriaceous lenses. Addition of glycerol did not decrease the CCT. Lens epithelial cells from eyes preserved in tap water for 48 hours appeared to be intact but lost cell organization.

Conclusions: Of the 15 tested methods to preserve porcine eyes, tap water (mineral content 1.79 mmol/L) resulted in the least corneal swelling. It is not necessary to use more expensive anterior chamber infusion fluids or other salt solutions.

8.1 Introduction

Wet laboratory (wet lab) training has a major role in teaching surgical competences to ophthalmic residents (Ament and Henderson, 2011, Lee et al., 2007). In 2006, 98% of ophthalmology residency programs in the United States were equipped with a microsurgery laboratory (Binenbaum and Volpe, 2006). It is important for ophthalmic

residents to practice surgical competences by repetition in a nonstressful setting (Dreyer and Volpe, 2000, Henderson and Ali, 2007).

Different eye models can be used for training ocular surgery; these include virtual eyes created by computer simulation, human donor eyes, manufactured artificial eyes, and animal eyes. Today, virtual reality can be used for training in different steps of cataract and vitreoretinal surgery; however, not all steps of these surgeries have been programmed and with this training, there is a lack of haptic feedback (Doyle et al., 2008, Selvander and Aman, 2012). Human donor eyes give the best resemblance to human cataract surgery in patients; however, there are problems with corneal transparency and availability (Auffarth et al., 1996, Borirak-chanyavat et al., 1995, Lenart et al., 2003, Liu et al., 2001, Smith, 2005). Another option is to work with manufactured eyes (Henderson et al., 2009). These eyes can help in the training of specific steps of cataract surgery; however, because they are made of silicone or other plastic material, they do not respond like eye tissues. A frequently used alternative is an animal model. Different animal models have been described, including bovine (Coroneo, 1990), porcine (Hashimoto et al., 2001, Saraiva and Casanova, 2003, Shentu et al., 2009, Sugiura et al., 1999, van Vreeswijk and Pameyer, 1998), ovine (Mohammadi et al., 2011), caprine (Sudan et al., 2002), and canine (Tolentino and Liu, 1975). The porcine eye model is the most frequently used model in wet lab training (Henderson et al., 2009). Porcine eyes are easy to obtain from local slaughterhouses; however, the elasticity of the lens capsule and the softness of the lens do not resemble the situation in the human cataractous lens (Sanchez et al., 2011, Sharma et al., 2011). Therefore, different approaches to induce cataract and stiffen the anterior lens capsule in porcine eyes have been described (Hashimoto et al., 2001, Saraiva and Casanova, 2003, Shentu et al., 2009, Sugiura et al., 1999, van Vreeswijk and Pameyer, 1998). In addition to the use for wet lab training, the porcine eye has also been described as a model for research in the fields of cataract surgery (Koopmans et al., 2004), retina (Taylor et al., 2013, Umazume et al., 2012), glaucoma (Ruiz-Ederra et al., 2005), and cornea research (Avila et al., 2012, Chen et al., 2012).

Working with porcine eyes is the best when they are as fresh as possible because the cornea starts to swell directly after enucleation. This corneal swelling gradually decreases the visibility of structures in the anterior chamber. An increase in corneal edema may cause unnecessary difficulties for trainee surgeons (Auffarth et al., 1996, Borirak-chanyavat et al., 1995, Lenart et al., 2003, Liu et al., 2001, Smith, 2005).

Because porcine eyes are obtained from local slaughterhouses, the planning of training and experiments depends on the schedule of the slaughterhouse because smaller slaughterhouses may not provide fresh eyes every day. In addition, planning an individual training program or an experiment can be difficult due to other, mostly clinical, duties. Thus, it would be beneficial if the eyes could be preserved for a longer period. We searched for a method to preserve porcine eyes for longer than 1 day and with the smallest increase in corneal thickness.

8.2 Materials and methods

Seventy-five natural porcine (*Sus scrofa domesticus*) eyes were obtained from the local slaughterhouse distributed over 6 weeks of testing. Central corneal thickness (CCT) measurements were performed within 6 hours post-mortem, at 22 to 24 hours and at 46 to 50 hours.

Preservation methods

After the first CCT measurements, the eyes were preserved for 46 to 50 hours in a liquid solution or without a solution in a closed environment. The solutions we used were phosphate-buffered saline (PBS), enriched balanced salt solution (BSS Plus),[A] balanced salt solution (Endosol),[B] purified water (mineral content 0.004 mmol/L),[C] mineral water with low mineral content (mineral content 0.058 mmol/L),[D] mineral water with high mineral content (mineral content 10.6 mmol/L),[E] and local tap water (mineral content 1.79 mmol/L).[F] The solution was put in a cup containing 3 eyes, and the cup was covered with a plastic sealing film.[G] The 15 eyes that were kept without a solution in a closed environment with air (air groups) were placed in a cup with the corneas facing each other. Between the CCT measurements, the cups were stored at +5 °C.

Three of the aforementioned preservation methods (NaCl 0.9%, tap water, and air) were also tested in combination with a glycerol treatment.[H] In these groups, the glycerol gel was applied on the cornea and left for 10 minutes. Then, the glycerol was wiped off, the cornea was flushed, and the CCT was measured.

After the poor results of preservation in air and favorable results of preservation in tap water were noticed, it was decided to include a group that started with preservation in air. After 22 hours of preservation, the preservation method was changed to tap water.

The goal was to determine whether the initial corneal swelling in an unfavorable condition could be reduced by preservation under a better condition (air → tap water group).

Central corneal thickness measurements

The CCT was measured by ultrasound (US) (Sonomed A-5500, Sonomed Escalon) using the A-scan with the speed of sound set at 1560 m/s (He and Liu, 2009). For the measurements, the eye was placed in a tray containing the preservation solution. If the eye was preserved in air, it was measured in a tray containing tap water.

The eye was placed on the bottom of the tray, resulting in a solution column of at least 1 cm above the cornea. The US probe was inserted in the solution, focused vertically on the center of the cornea, and the thickness of the cornea was measured. To reduce the influence of within-measurement variation, 6 thickness measurements of each cornea were performed per day.

The first measurements were performed within 6 hours post-mortem. After the first measurements, the eyes were placed in the cups for preservation. The second measurements were performed after 22 to 24 hours of preservation, and the third measurements were finished within 46 to 50 hours of preservation.

Lens epithelial cell survival

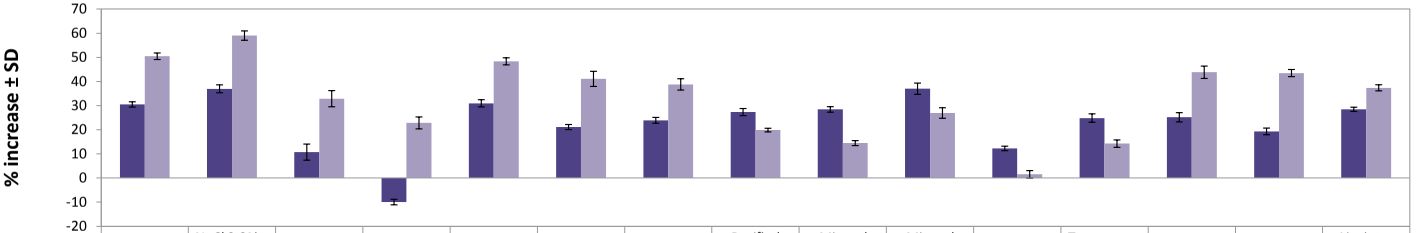
In addition to taking CCT measurements, study goal was to determine the integrity of the lens epithelial cells (LECs) after preservation. To observe whether the LECs were still intact after a preservation period of 46 to 50 hours, 6 lenses were extracted from the eyes and the LECs were stained. Three lenses from eyes within 6 hours post-mortem and 3 lenses from eyes preserved in tap water for 48 hours were extracted. To extract the lens, the cornea was cut off with scissors with sharp curved blades. Next, the iris was removed and the zonule was torn using blunt Westcott scissors. Then, the attached vitreous humor was removed by lifting the lens with the scissors. After extraction, the lenses were fixed with paraformaldehyde (concentration 3.7%) for 2 hours. Then, the lenses were extracted with 0.5% Triton X-100 in PBS for 3 minutes. Subsequently, they were incubated for 30 minutes with TRITC-labeled phalloidin in a final concentration of 2 mg/mL and 4´,6-Diamidino-2-phenylindole, dihydrochloride in a final concentration of 4 mg/mL dissolved in PBS with 1% bovine serum albumin (PBSA). Finally, the lenses were washed (2 times in PBSA and 1 time in PBS) and stored in PBS until microscopic observation. For imaging, a confocal microscope was used (TCS SP2, Leica Microsystems GmbH).

Outcome variables

For each preservation method, 3 or 6 corneas were measured. Each cornea was measured 6 times per day. This resulted in 18 or 36 measurements per preservation method. The mean and standard deviation (SD) were calculated for the CCTs with the different preservation methods, resulting in a mean and SD per method per day. Of these means, the relative difference was calculated. The relative difference was the percentage increase in CCT compared with the first measurements. This results in two relative differences. The first is the relative difference between the first and second measurement, and the second is the relative difference between the first and third measurement. These relative differences were used to show the relative relationships between the preservation methods.

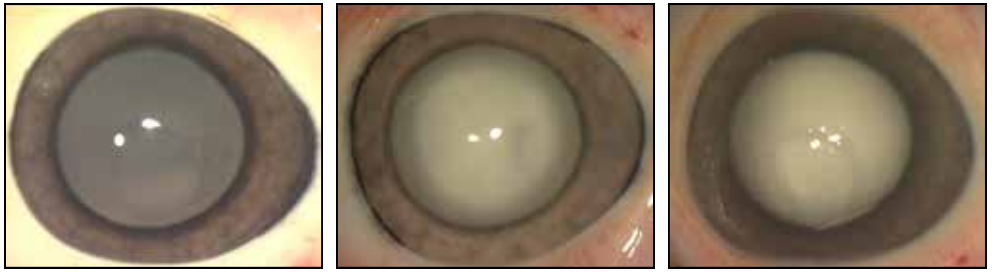
Statistical analysis

Data were analyzed using SPSS software (version 20, International Business Machines Corp.). The data were analyzed in three ways. First, for each preservation method a comparison was made of the CCT between the 3 days and 6 measurements per cornea using repeated-measures multivariate analyses of variance (MANOVA). The first measurements were compared with the second measurements (22 to 24 hours of preservation) and the first measurements with the third measurements (46 to 50 hours of preservation). To examine the effect of the different preservation methods over the 3 days and 6 measurements per cornea, repeated-measures MANOVA with a Games-Howell post-hoc analysis was used. Finally, the effect of the addition of glycerol and the effect of the change from preservation in air to preservation in tap water were evaluated with repeated-measures MANOVA. Data in text are given as the mean and standard error unless otherwise stated. A P-value less than 0.05 was considered statistically significant.



	NaCl 0.9%	NaCl 0.9% + glycerol	NaCl 5%	NaCl 10%	PBS	BSS plus	Endosol	Purified water	Mineral water low	Mineral water high	Tap water	Tap water + glycerol	Air	Air + glycerol	Air → tap water
22-24 hours	30.49	36.97	10.73	-9.96	30.96	21.12	23.90	27.32	28.42	37.01	12.26	24.84	25.16	19.31	28.46
46-50 hours	50.45	59.01	32.87	22.83	48.34	41.08	38.79	19.86	14.46	26.94	1.54	14.25	43.82	43.48	37.37

FIGURE 1. *Relative differences in CCT for the different preservation methods (NaCl = sodium chloride; PBS = phosphate-buffered saline).*



Preserved in NaCl 0.9% Preserved in NaCl 5% Preserved in NaCl 10%

FIGURE 2. *Images of eyes preserved in NaCl 0.9%, NaCl 5%, and NaCl 10% for 22 hours. The photographs were taken directly after the US measurements (NaCl = sodium chloride).*

TABLE 1. *Central corneal thickness at day 0, day 1, and day 2*

		Central corneal thickness (mm)		
		day 0	day 1	day 2
Method	N	Mean (SE)	Mean (SE)	Mean (SE)
NaCl 0.9%	6	0.88 (0.02)	1.15 (0.03)	1.32 (0.04)
NaCl 0.9% + glycerol	6	0.83 (0.02)	1.13 (0.03)	1.31 (0.04)
NaCl 5%	3	0.86 (0.03)	0.95 (0.04)*	1.14 (0.05)
NaCl 10%	3	0.88 (0.03)	0.79 (0.04)*	1.08 (0.05)
PBS	6	0.90 (0.02)	1.18 (0.03)	1.33 (0.04)
BSS plus	3	0.94 (0.03)	1.14 (0.04)	1.32 (0.05)
Endosol	3	0.92 (0.03)	1.14 (0.04)	1.28 (0.05)
Purified water	6	0.84 (0.02)	1.07 (0.03)	1.01 (0.04)
Mineral water low	6	0.89 (0.02)	1.14 (0.03)	1.02 (0.04)*
Mineral water high	6	0.87 (0.02)	1.19 (0.03)	1.10 (0.04)
Tap water	6	0.87 (0.02)	0.98 (0.03)	0.88 (0.04)*
Tap water + glycerol	6	0.87 (0.02)	1.06 (0.03)	0.92 (0.04)*
Air	6	0.91 (0.02)	1.14 (0.03)	1.31 (0.04)
Air + glycerol	3	1.04 (0.03)	1.24 (0.04)	1.49 (0.05)
Air → tap water	6	1.18 (0.02)	1.11 (0.03)	1.18 (0.04)

The differences in central corneal thickness were compared for day 0 - day 1 and day 0 - day 2, not significant differences are indicated with *.

8.3 Results

Central corneal thickness in each preservation method

Table 1 shows the mean CCT for each preservation method per day. For most preservation methods, with exception of the tap water, NaCl 5%, NaCl 10%, and mineral water with low mineral content groups, there was a statistically significant increase in the mean CCT over the 3 days ($P \leq 0.032$). However, in eyes preserved in mineral water with low mineral content and tap water (also with the addition of glycerol), there was no statistically significant difference in CCT between day 0 and day 2 ($P = 0.123$, $P = 0.731$, and $P = 0.191$, respectively). The same applied to the NaCl 5% and NaCl 10% groups for the CCT between day 0 and day 1 ($P = 0.238$ and $P = 0.079$, respectively).

Comparison of preservation methods

Figure 1 shows an overview of the relative differences between the preservation methods. There was a statistically significant interaction effect between the preservation method

and the CCT over 3 days ($F[20, 86] = 14.90$, $P < 0.001$). The interaction effect between the preservation method, the CCT over 3 days, and the CCT in the 6 repeated measurements was not statistically significant ($F[100, 430] = 1.25$, $P = 0.079$). This can be explained by the 6 repeated CCT measurements per day being independent from one another. The main effect of the variable measurement was also not statistically significant in the model ($F[5, 215] = 2.03$, $P = 0.076$).

Table 2 compares the preservation methods. Games-Howell post-hoc tests showed that the CCT in the NaCl 10% and tap water groups increased significantly less than with four of the other preservation methods and that the CCT in the purified water group increased statistically significantly less than with the two other methods. For example, in Table 2, the mean difference between the tap water and air group was 0.204 ± 0.028, corresponding with $P = 0.001$ from the post-hoc tests. Even though the NaCl 10% preservation method showed good results for the CCT measurements, preservation of eyes in the NaCl solutions also stiffened the lens. This resulted in shrunken, coriaceous lenses with a surrounding layer of fluid (Figure 2). The addition of glycerol did not lead to a significant decrease in CCT compared with the same preservation methods without glycerol (Figure 1). In the NaCl 0.9% group, the Mauchly test indicated that the assumption of sphericity had been violated for the main effect of days ($\chi^2[2] = 14.98$, $P = 0.001$). Therefore, the degrees of freedom were corrected in this group using Greenhouse-Geisser estimates of sphericity ($\varepsilon = 0.55$). In the NaCl 0.9% group, there was no statistically significant interaction effect between glycerol and the CCT over the 3 days ($F[1.11, 11.05] = 1.75$, $P = 0.214$). In the tap water group, the interaction effect between glycerol and CCT over the 3 days was $F(2, 20) = 1.68$ and $P = 0.211$. In the air group, the interaction effect was $F(2, 14) = 1.03$ and $P = 0.381$. Thus, there was no significant interaction between the glycerol and the CCT in these 3 tested methods.

In the air → tap water group, the preservation method was changed to tap water after 22 hours of preservation. To determine whether this change to tap water provided a decrease in CCT, the air → tap water group was compared with the air group. There was no significant interaction between the air group and the air → tap water group when the CCT at day 0 was compared with the CCT at day 2 (after the change to tap water) ($F[1, 10] = 3.81$, $P = 0.080$). The interaction between the two groups was also not statistically significant when day 0 was compared with day 1 (before the change to tap water) ($F[1, 10] = 0.235$, $P = 0.638$). This means that the CCT measurements before the change to tap water were not different between the two groups and that there was no effect on the CCT when the preservation method was changed from air to tap water.

TABLE 2. *Comparison of differences between the preservation methods*

	Mean difference (SE)									
Method	NaCl 0.9%	NaCl 5%	NaCl 10%	PBS	BSS plus	Endosol	Purified	Mineral L	Mineral H	Tap water
NaCl 0.9%	-									
NaCl 5%	.129 (.055)	-								
NaCl 10%	.196 (.026)*	.067 (.051)	-							
PBS	.022 (.031)	.151 (.054)	.218 (.024)†	-						
BSS plus	.022 (.053)	.151 (.068)	.218 (.049)	.000 (.052)	-					
Endosol	.005 (.033)	.124 (.055)	.191 (.026)*	.027 (.032)	.027 (.053)	-				
Purified water	.141 (.035)	.012 (.056)	.056 (.029)	.163 (.034)*	.162 (.054)	.136 (.035)	-			
Mineral L	.098 (.046)	.031 (.064)	.098 (.041)	.120 (.045)	.120 (.062)	.093 (.046)	.042 (.047)	-		
Mineral H	.061 (.041)	.068 (.060)	.135 (.036)	.083 (.040)	.083 (.058)	.056 (.041)	.079 (.043)	.037 (.052)	-	
Tap water	.202 (.029)*	.073 (.052)	.006 (.021)	.224 (.027)†	.224 (.051)	.197 (.029)	.062 (.032)	.104 (.043)	.141 (.038)	-
Air	.002 (.032)	.131 (.054)	.198 (.025)*	.020 (.031)	.020 (.052)	.007 (.035)	.142 (.035)*	.100 (.045)	.063 (.041)	.204 (.028)†

Mineral L stands for the mineral water group with low mineral content. Mineral H stands for mineral water group with high mineral content.
* Significant difference with P value ≤ 0.05
† Significant difference with P value ≤ 0.001

Confocal imaging

The LECs were still intact after preservation in tap water for 48 hours (Figure 3B). However, compared with the LECs in Figure 3A, there was a difference in cell organization. The LECs from the preserved lenses were intact but lost their general structure. This can be seen from the larger internuclear spaces in the left image in Figure 3B. Also the cytoskeleton of the lens fibers on the right bottom image in Figure 3B, has a more disorganized arrangement.

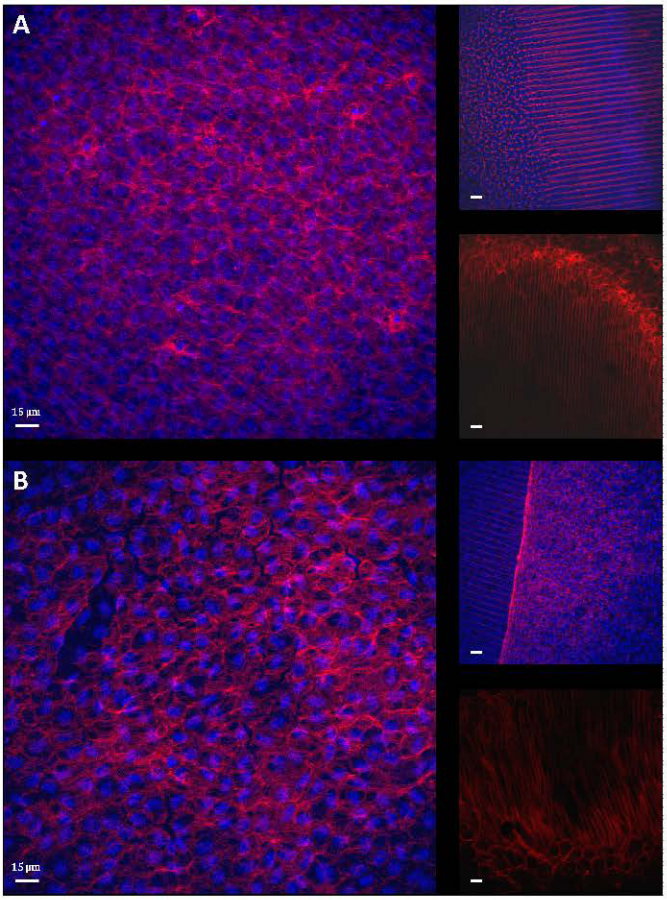


FIGURE 3. *Confocal laser scanning microscope images of an eye that was not preserved and extracted directly after it was obtained at the slaughterhouse (A) and from an eye preserved in tap water and extracted after 48 hours of preservation (B). In both figures, the left image shows the anterior LECs, the right upper image represents the cells in the equatorial region, and the right bottom image is the posterior view.*

8.4 Discussion

For planning wet lab training and experiments, it would be beneficial if the porcine eyes could be preserved for a certain period of time without the cornea swelling. The present study found that tap water was an appropriate preservation solution for porcine corneas. Eyes preserved in tap water maintained their corneal transparency and thickness for up to 50 hours.

With preservation in the hyperosmotic NaCl 10% solution, the corneas also maintained their natural thickness. However, this was accompanied by stiffening of the lens, resulting in shrunken, coriaceous lenses with a surrounding layer of fluid. This made the lenses unusable for wet lab training or experiments.

The preservation of porcine eyes has not been researched extensively. Igarashi et al. (Igarashi et al., 1993) compared three solutions for the preservation of porcine corneas for 24 hours. The corneal integrity was measured by nuclear magnetic resonance (NMR) spectroscopy, and bicarbonate-free glucose-phosphate Ringer solution gave the best results. Saline and Ringer lactate were also tested; however, these preservation methods led to a larger decrease in the peaks of the NMR spectrum than the first solution. Another study by Swinger and Kornmehl in 1985 (Swinger and Kornmehl, 1985) described a method to dehydrate the cornea of post-mortem human and animal eyes. They removed the corneal epithelium and filled the anterior chamber with a solution containing 30% dextran, 0.05% methylparaben, and 0.005% propylparaben in saline. This solution improved corneal transparency and decreased the CCT to approximately 0.45 mm; however, no baseline value was given and there was no description of which animal models were used.

Few studies of corneal transparency have been performed using corneas of human donor eyes. Liu et al. (2001) found that the use of glycerol in the anterior chamber decreased corneal transparency. Auffarth et al. (1996) report that a clear cornea can be achieved for approximately 4 hours by injecting a hyperosmotic dextran solution into the anterior chamber. This is the same solution studied by Swinger and Kornmehl (1985). Use of this solution permits human donor eyes to be used for wet lab training for up to 4 days post-mortem. Furthermore, artificial corneas have been used to circumvent the problem of decreased corneal transparency (Borirak-chanyavat et al., 1995, Lenart et al., 2003). However, the use of artificial corneas in a wet lab entails extra steps and is often time consuming. Other studies examined how to protect the cornea from a decrease in transparency when a fixative was used to induce cataract in porcine eyes (Hashimoto et al., 2001, Saraiva and Casanova, 2003, Sugiura et al., 1999). In these studies, methods were

described to protect the cornea from contact with the fixative by injecting a viscoelastic material into the anterior chamber. Nevertheless, none of the aforementioned studies evaluated the wide range of preservation methods described in our study.

The present study has limitations. We aimed to evaluate the transparency of the corneas. However, we did not directly measure the transparency; instead we used the CCT measured by A-scan US to compare the different preservation methods. Nevertheless, this is a representative comparison because it is known that corneal swelling can translate into a decrease in corneal transparency (Jester et al., 2001, Maurice, 1957).

We performed confocal imaging of lenses only of eyes preserved in tap water because this proved to be the best preservation method regarding the CCT measurements. Perhaps for the preservation of eyes where survival of LECs is important, other preservation methods are more suitable.

In this study, 15 preservation methods were tested; however, there are many more possible preservation methods. First, we selected methods that are easily available and applicable in a wet lab, such as tap water, NaCl 0.9%, PBS, and air. The other methods were selected because we found evidence that they have an effect on CCT and corneal transparency. As mentioned, the use of glycerol was described by Liu et al. (2001). They used glycerol in the anterior chamber of human cadaver eyes to reduce corneal edema. Their experience was that topical glycerol was not effective; however, this finding was not further described. Because of the osmotic properties of glycerol, we thought it would be of interest to test glycerol in combination with other preservation methods. However, we did not find a notable benefit to using glycerol.

After noticing poor results of preservation in air and favorable results of preservation in tap water, it was decided to include a group that started with preservation in air and changed to tap water after 22 hours of preservation. We wanted to determine whether the effects of initial preservation in an unfavorable condition could be improved by preservation under a better condition. Nevertheless, we did not find an improvement in the CCT by the change to tap water.

In addition, we had expected that anterior chamber irrigation fluids such as BSS Plus and Endosol would provide the best environment for the corneas because these fluids are designed to maintain the corneal integrity during cataract surgery. However, the CCT increased with preservation in anterior chamber irrigation fluids. These irrigation fluids are designed for human eyes, not for porcine eyes. Furthermore, these fluids were developed for irrigation of the anterior chamber, whereas we used them as a topical preservation method for the whole eye.

Next, we tested the NaCl 5% and NaCl 10% solutions as preservation methods because of their hyperosmotic properties as the use of NaCl 0.9% caused an increase in CCT. We found the expected maintenance in CCT in the NaCl 10% group. Nevertheless, due to changes in the structure of the lens, these hyperosmotic NaCl solutions are not ideal for preservation.

Finally, purified water and mineral waters were selected because we found a benefit to using tap water and we wanted to compare tap water with a standardized formulation of waters with different mineral contents. In the repeated-measures MANOVA, there was no significant difference in CCT between these waters and tap water. However, as the bars in Figure 1 show, the relative difference in CCT was the lowest in the tap water group.

The difference between local tap water, purified water, and mineral water is the mineral content. The beneficial effect of our local tap water on the CCT may be related to this specific mineral content. Tap water in different regions consists of varying mineral contents, and the mineral content of the tap water used in our study was 1.79 mmol/L. This mineral content is quite high compared with that in other regions in the Netherlands. This implies that tap water from other regions might give different results. The mineral content of mineral waters available at the supermarkets in the Netherlands is lower than that of tap water (Azoulay et al., 2001), and we have not found a commercially available alternative to our local tap water. However, we also tested a mineral water[E] that is commercially available in Belgium and France and contains a substantially higher mineral content than our local tap water. Nevertheless, preservation in mineral water with a high mineral content did not result in thinner corneas than preservation in our local tap water. Because our tap water is only available locally, trainee surgeons living elsewhere might consider using purified water for the preservation of porcine eyes. Purified water is usually available in laboratories worldwide, and in this study the increase in CCT was relatively low when purified water was used. In addition, the results with purified water were not significantly different from those with tap water. Mineral water also led to relatively low increases in CCT.

In conclusion, we found that tap water may be an appropriate method to preserve porcine eyes. Furthermore, our results show that for the preservation of porcine eyes, it is not necessary to use more expensive anterior chamber infusion fluids or other salt solutions. Finally, the result that porcine eyes can be preserved for up to 50 hours provides some flexibility in the time schedule or can save time. This can make planning wet lab training and experiments more manageable.

References

Ament C.S., Henderson B.A., 2011. Optimizing resident education in cataract surgery. Curr. Opin. Ophthalmol. 22, 64-67.

Auffarth G.U., Wesendahl T.A., Solomon K.D., Brown S.J., Apple D.J., 1996. A modified preparation technique for closed-system ocular surgery of human eyes obtained postmortem: an improved research and teaching tool. Ophthalmology 103, 977-982.

Avila M.Y., Gerena V.A., Navia J.L., 2012. Corneal crosslinking with genipin, comparison with UV-riboflavin in ex-vivo model. Mol. Vis. 18, 1068-1073.

Azoulay A., Garzon P., Eisenberg M.J., 2001. Comparison of the Mineral Content of Tap Water and Bottled Waters. J. Gen. Intern. Med. 16, 168-175.

Binenbaum G., Volpe N.J., 2006. Ophthalmology resident surgical competency: a national survey. Ophthalmology 113, 1237-1244.

Borirak-chanyavat S., Lindquist T.D., Kaplan H.J., 1995. A cadaveric eye model for practicing anterior and posterior segment surgeries. Ophthalmology 102, 1932-1935.

Chen S., Li Y., Stojanovic A., Zhang J., Wang Y., Wang Q., Seiler T., 2012. Evaluation of the efficacy of excimer laser ablation of cross-linked porcine cornea. PLoS One 7, e46232.

Coroneo M.T., 1990. The bovine eye as a model for the novice cataract surgeon. Ophthalmic Surg. 21, 772-777.

Doyle L., Gauthier N., Ramanathan S., Okamura A., 2008. A simulator to explore the role of haptic feedback in cataract surgery training. Stud. Health Technol. Inform. 132, 106-111.

Dreyer E.B., Volpe N.J., 2000. Eleven commandments for teaching ophthalmic surgery. Ophthalmology 107, 415-416.

Hashimoto C., Kurosaka D., Uetsuki Y., 2001. Teaching continuous curvilinear capsulorhexis using a postmortem pig eye with simulated cataract. J. Cataract Refract. Surg. 27, 814-816.

He X., Liu J., 2009. A quantitative ultrasonic spectroscopy method for noninvasive determination of corneal biomechanical properties. Invest. Ophthalmol. Vis. Sci. 50, 5148-5154.

Henderson B.A., Ali R., 2007. Teaching and assessing competence in cataract surgery. Curr. Opin. Ophthalmol. 18, 27-31.

Henderson B.A., Grimes K.J., Fintelmann R.E., Oetting T.A., 2009. Stepwise approach to establishing an ophthalmology wet laboratory. J. Cataract Refract. Surg. 35, 1121-1128.

Igarashi H., Yoshida A., Tanaka K., 1993. Comparison of pig cornea preservation solutions using quantitative 31P NMR spectroscopy. Magn. Reson. Med. 29, 107-109.

Jester J.V., Ghee Lee Y., Li J., Chakravarti S., Paul J., Petroll W.M., Dwight Cavanagh H., 2001. Measurement of corneal sublayer thickness and transparency in transgenic mice with altered corneal clarity using in vivo confocal microscopy. Vision Res. 41, 1283-1290.

Koopmans S.A., Terwee T., Haitjema H.J., Deuring H., Aarle S., Kooijman A.C., 2004. Relation between injected volume and optical parameters in refilled isolated porcine lenses. Ophthalmic Physiol. Opt. 24, 572-579.

Lee A.G., Greenlee E., Oetting T.A., Beaver H.A., Johnson A.T., Boldt H.C., Abramoff M., Olson R., Carter K., 2007. The Iowa ophthalmology wet laboratory curriculum for teaching and assessing cataract surgical competency. Ophthalmology 114, e21-26.

Lenart T.D., McCannel C.A., Baratz K.H., Robertson D.M., 2003. A contact lens as an artificial cornea for improved visualization during practice surgery on cadaver eyes. Arch. Ophthalmol. 121, 16-19.

Liu E.S., Eng K.T., Braga-Mele R., 2001. Using medical lubricating jelly in human cadaver eyes to teach ophthalmic surgery. J. Cataract Refract. Surg. 27, 1545-1547.

Maurice D.M., 1957. The structure and transparency of the cornea. J. Physiol. 136, 263-286.

Mohammadi S.F., Mazouri A., Jabbarvand M., Rahman-A N., Mohammadi A., 2011. Sheep practice eye for ophthalmic surgery training in skills laboratory. J. Cataract Refract. Surg. 37, 987-991.

Ruiz-Ederra J., Garcia M., Hernandez M., Urcola H., Hernandez-Barbachano E., Araiz J., Vecino E., 2005. The pig eye as a novel model of glaucoma. Exp. Eye Res. 81, 561-569.

Sanchez I., Martin R., Ussa F., Fernandez-Bueno I., 2011. The parameters of the porcine eyeball. Graefes Arch. Clin. Exp. Ophthalmol. 249, 475-482.

Saraiva V.S., Casanova F.H., 2003. Cataract induction in pig eyes using viscoelastic endothelial protection and a formaldehyde-methanol mixture. J. Cataract Refract. Surg. 29, 1479-1481.

Selvander M., Aman P., 2012. Virtual reality cataract surgery training: learning curves and concurrent validity. Acta Ophthalmol. 90, 412-417.

Sharma P.K., Busscher H.J., Terwee T., Koopmans S.A., van Kooten T.G., 2011. A comparative study on the viscoelastic properties of human and animal lenses. Exp. Eye Res. .

Shentu X., Tang X., Ye P., Yao K., 2009. Combined microwave energy and fixative agent for cataract induction in pig eyes. J. Cataract Refract. Surg. 35, 1150-1155.

Smith J.H., 2005. Teaching phacoemulsification in US ophthalmology residencies: can the quality be maintained? Curr. Opin. Ophthalmol. 16, 27-32.

Sudan R., Titiyal J.S., Sethi H.S., Rai H.K., 2002. Animal model for teaching phacoemulsification. J. Cataract Refract. Surg. 28, 4-5.

Sugiura T., Kurosaka D., Uezuki Y., Eguchi S., Obata H., Takahashi T., 1999. Creating cataract in a pig eye. J. Cataract Refract. Surg. 25, 615-621.

Swinger C.A., Kornmehl E.W., 1985. Dehydration of post-mortem eyes for practice and experimental surgery. Ophthalmic Surg. 16, 182-183.

Taylor L., Arner K., Engelsberg K., Ghosh F., 2013. Effects of Glial Cell Line-derived Neurotrophic Factor on the Cultured Adult Full-thickness Porcine Retina. Curr. Eye Res. 38, 503-515.

Tolentino F.I., Liu H.S., 1975. A laboratory animal model for phacoemulsification practice. Am. J. Ophthalmol. 80, 545-546.

Umazume K., Barak Y., McDonald K., Liu L., Kaplan H.J., Tamiya S., 2012. Proliferative vitreoretinopathy in the Swine-a new model. Invest. Ophthalmol. Vis. Sci. 53, 4910-4916.

van Vreeswijk H., Pameyer J.H., 1998. Inducing cataract in postmortem pig eyes for cataract surgery training purposes. J. Cataract Refract. Surg. 24, 17-18.

Other cited material

A. Alcon Laboratories, Inc. BSS Plus [package insert]. Available at: http://ecatalog.alcon.com/PI/BSSplus500_us_en.pdf. Accessed November 9, 2013
B. AbbottMedicalOptics, Inc.AMO_ Endosol balanced salt solution for irrigation [package insert]. Available at: http://www.dcmedical.be/files/client/1053/ftp/AMO_Endosol_Product_fiche.pdf. Accessed November 9, 2013
C. EMD Millipore Corp. Milli-Q [label]
D. Spadel Group. Spa Reine [label]. Available at: http://www.bottledwaterweb.com/bottlersdetail.do?kZ70. Accessed November 9, 2013
E. Nestlé Waters. Vittel Grande Source [label]. Available at: http://en.wikipedia.org/wiki/Vittel_(water). Accessed November 9,2013
F. Waterbedrijf, Groningen, the Netherlands. Mineral content. Available at: http://www.waterbedrijfgroningen.nl/nl/water-thuis/Klantenservice/veelgestelde-vragen/waterkwaliteit/Paginas/Watziterinhetwater.aspx. Accessed November 9, 2013
G. Pechiney Plastic Packaging Co. Parafilm [package insert]
H. Johnson & Johnson. K-Y Jelly [package insert]

Chapter 9

Influence of stereoscopic vision on task performance with the operating microscope

Lisanne M. Nibourg, Wouter Wanders, Frans W. Cornelissen, and Steven A. Koopmans

This chapter is an edited version of the manuscript: Nibourg L.M., Wanders W., Cornelissen F.W., Koopmans S.A., 2015. Influence of stereoscopic vision on task performance with the operating microscope. Journal of Cataract and Refractive Surgery, Epub. (Re-use permitted)

Abstract

Purpose: To determine to what extent stereoscopic depth perception influences performance of tasks executed under an operating microscope.

Setting: Laboratory of Experimental Ophthalmology, University Medical Center Groningen, the Netherlands.

Design: Experimental study.

Methods: Medical students were assigned (on the basis of their stereoacuity) to a stereo-sufficient group (depth perception ≤240 seconds of arc [arcsec]) or stereo-deficient group (≥480 arcsec). They performed a bead-stringing task (a mockup surgical test) under an operating microscope or a task on a cataract surgery simulator. The stereo-sufficient subjects also performed the bead-stringing task under artificial stereo-deficient conditions (binocular and monocular viewing).

Results: The study comprised 77 medical students. The stereo-sufficient subjects performed both tasks faster than the stereo-deficient subjects and artificially stereo-deficient subjects ($P \leq 0.024$). In addition, a within-group analysis established that the stereo-sufficient subjects were faster at the bead-stringing task with stereoscopic viewing than under artificial stereo-deficient conditions with binocular viewing ($P \leq 0.011$).

Conclusions: Having stereovision resulted in better initial performance on certain tasks involving the use of an operating microscope or cataract surgery simulator. However, this study did not show that stereo deficiency necessarily results in an inability to perform such tasks properly. Hence, it was not evident that for admission to an ophthalmology residency program, stereovision should be judged more stringently than other traits.

9.1 Introduction

Achieving a minimum score on a standardized random-dot stereoscopy test has been part of the admission requirements for training in the specialty of ophthalmology in the

Netherlands since 2001.[A] In other countries, however, ophthalmology training programs, as well as training programs for other medical specialties that also use stereoscopic operating microscopes, do not select applicants on the basis of stereoacuity (Wong et al., 2010). Moreover, there is little to no evidence that performance with such a microscope would suffer from a lack of stereovision. This lack of evidence raises the question of whether the performance of young physicians using a surgical microscope would be influenced by a lack of stereovision.

Since 1921, microscopes have been used in medical operative practice (Nylen, 1972). The first surgical microscopes did not provide the same image quality as those of today, and many improvements followed. At present, operating microscopes are equipped with stereoscopic binoculars, which allow the perception of depth However, depth perception under an operating microscope is available only to surgeons who have sufficient stereovision. Between 1980 and 1995, difficulties in the surgical training of some ophthalmology residents were reported.[A] The difficulties concerned the residents' operative skills and were attributed to their lack of stereovision. Despite the absence of direct scientific evidence, it was presumed that a relationship existed between the lack of stereoacuity and moderate operative skills in using the stereoscopic microscope. This hypothesis resulted in stereoacuity requirements for training in the specialty of ophthalmology in the Netherlands. It has been reported that besides the Netherlands, only the Czech Republic has requirements regarding stereoacuity for ophthalmic surgeons (Wong et al., 2010).

The aim of this study was to determine the extent to which stereoscopic depth perception influences the performance of tasks executed under an operating microscope. Therefore, an experiment with a mockup surgical test (bead-stringing task) and a cataract surgery simulator was designed.

9.2 Material and methods

Subjects

Students enrolled in the master of medicine program at University Medical Center Groningen were recruited to participate in the study. The study subjects included students who were participating in an internship in ophthalmology as well as students in other departments. The subjects had to state that they had normal function in both hands.

Ophthalmic measurements

Corrected distance visual acuity (CDVA) was measured with the Freiburg Visual Acuity Test (Bach, 2007). Because of the relationship between visual acuity and stereoacuity, only subjects with a CDVA of better than 20/20 were included (Cagenello et al., 1993, Donzis et al., 1983, Goodwin and Romano, 1985). Stereoacuity was assessed with the TNO Stereotest (Laméris Ootech BV) (Okuda et al., 1977). On the basis of the stereotest results, subjects were divided into 2 groups. The first group of subjects had a stereoscopic depth perception of 240 seconds of arc (arcsec) or less (the stereo-sufficient group). This criterion was based on the prerequisites for the ophthalmology training program in the Netherlands. The second group of subjects was considered stereoblind; they had a stereoscopic depth perception of 480 arcsec or more or were unable to see any figures during the stereotest (the stereo-deficient group).

Bead stringing task (mock-up surgical test)

For this task, an operating microscope (OPMI 6-SFC, Carl Zeiss Meditec AG) was used. Subjects had to string small beads (rocailles, size 15/0) onto a 6-0 nylon suture (Ethilon) with a forceps and a needle holder. The beads (25 black and 25 white) were provided in a tray under the microscope. They had to be strung onto the suture to create a chain of alternating black and white beads. Because not all the beads would fit on the needle, subjects had to slip the beads from the needle to the suture thread after each set of 5 beads. The task was completed once 20 beads were on the thread.

For this task, 3 viewing conditions were established as follows: with a stereoscopic viewer, with a binocular viewer without stereoscopic vision, and with a monocular viewer. These viewing conditions were created on the operating microscope with 2 sets of oculars; that is, a set with stereoscopic view and a set without stereoscopic view. Under the monocular viewing condition, subjects selected an ocular (from the set without stereoscopic view) to be covered. The stereo-sufficient group had to perform the task 5 times in a row under 1 of the viewing conditions followed by 5 times in a row under another viewing condition, thus resulting in 10 runs per subject. The 3 different viewing conditions were used in 4 combinations to create 4 testing categories (Table 1). Stereo-sufficient subjects were assigned to 1 of these categories using stratified randomization (categories SS1 through SS4). The 2 stratification factors in this study were category and sex. Subjects in the stereo-deficient group had to perform the task 5 times in the stereoscopic viewing condition only (category SD1).

TABLE 1. *Overview of conditions in the bead stringing and cataract simulator tasks*

Task	Group	Category	Number of subjects	Viewing condition 1	Viewing condition 2
Bead stringing task	Stereo-sufficient (SS)	SS1	9	Stereo	Binocular
		SS2	9	Binocular†	Stereo
		SS3	8	Monocular	Binocular
		SS4	7	Binocular	Monocular
	Stereo-deficient (SD)	SD1	7	Stereo	-
Cataract surgery simulator task	Stereo-sufficient (SS)	SS5	28	Stereo	-
	Stereo-deficient (SD)	SD2	9	Stereo	-

In each viewing condition of the bead stringing task, subjects performed five runs in a row. In both the binocular and monocular viewing conditions, subjects were rendered stereoblind. In the simulator task, subjects performed five runs in a row. † The binocular viewing condition in the SS2 category is also named artificial SD group.

Simulator task

For this task, the Eyesi Surgical cataract surgery simulator and software (version 2.7, VRmagic Holding AG) were used. The simulator consisted of a model head connected to a microscope, which provided a virtual stereoscopic view of an eye lying under the operating microscope. The subjects (categories SS5 and SD2) had to execute the first level of the cataract forceps training 5 times. For this task, subjects had to grasp small cubes that were floating in the anterior chamber and drag them into a frame situated in the middle of the anterior chamber. When all the blocks had been placed within the circle, the task was completed. The time needed to execute the task was recorded.

Outcome parameters

Execution of the bead-stringing task was recorded with a digital camera attached to the microscope. For each separate run, the test time was determined from the recording by measuring the interval between the actual start and stop times. Errors made during execution of the task were scored as 1 point. Errors included beads running away, beads falling from the needle, and the needle falling from the needle holder.

To evaluate performance across the different viewing conditions, 2 outcome parameters were used. The first outcome parameter was the median test time of the last 3 attempts of each series. (To eliminate possible effects of learning during the initial attempts, only the last 3 attempts were considered.) Furthermore, the test time just before switching oculars was compared with that directly after switching viewing conditions (ie, the test time of run 5 [the last run under the first viewing condition] was compared with the test time of run 6 [the first run under the second viewing condition]).

During the simulator task, completion times of the 5 executions by each subject were recorded. The 5 completion times were used to determine each subject's median test time.

Statistical analyses

Statistical analyses were performed with Statistical Package for the Social Sciences software (version 20, International Business Machines Corp.). Four comparisons for the bead-stringing task and 1 comparison for the simulator task were performed. Table 2 shows the compared groups, outcome parameters, and statistical tests used in the analyses. For all tests, P values less than 0.05 were considered statistically significant.

TABLE 2. *Overview of statistical analyses performed per task and group*

Task	Figure	Comparison	Outcome parameters	Statistical test
Bead stringing task	1	Viewing condition 1: SS1 vs. SD1	Test time	Repeated measures analysis of variance (rANOVA)
	2	Viewing condition 1: SS1 vs. SS2 vs. SD1	Median test times	Kruskal-Wallis test, with Wilcoxon rank-sum post-hoc analysis and Holm-Bonferroni correction
	3	Within group analysis: viewing condition 1 vs. 2 (SS1, SS2, SS3 and SS4)	Median test times Switch of conditions	Wilcoxon rank-sum tests
	4	Correlation test time and errors (SS1, SS2, SS3, SS4 and SD1) and correlation SS1-4 vs. correlation SD1	Test time Errors	Spearman's correlation coefficients Fisher z-transformation
Cataract surgery simulator task	5	SS5 vs. SD2	Median test times	Wilcoxon rank-sum test

For both tasks, the compared groups and outcome parameters are given. Figures corresponding to the data are also indicated.

9.3 Results

Of the 77 study subjects, 40 were recruited for the bead-stringing task and 37 for the simulator task. The subjects had a mean age of 23.2 years (range 20 to 28 years), and 30 (39.0%) were men. Their CDVA ranged between 20/17 and 20/11, with a mean of 20/13. The stereo-sufficient group had a mean score of 33 arcsec on the TNO Stereotest (range 15 to 120 arcsec). In the stereo-deficient group, 2 subjects were able to detect a figure at 480 arcsec, whereas the others were unable to see any figures of the stereotest. Causes for stereoblindness were amblyopia and strabismus.

First, performance of the bead-stringing task by the stereo-sufficient subjects (SS1, condition 1 only) was compared with that by the stereo-deficient (SD1) subjects. For this comparison, test time was shown as a function of test number (Figure 1). A gradual

decrease in time required to complete the test was noted for both groups of subjects: $F(4,52) = 2.90$ and $P = 0.031$. Nevertheless, the stereo-deficient subjects remained slower than the stereo-sufficient group: $F(4,52) = 16.1$ and $P < 0.001$.

Next, the performance of the stereo-deficient subjects (SD1) was compared with that of the subjects rendered artificially stereo-deficient (group SS2, condition 1 only). For completeness, data for the stereo-sufficient subjects (SS1, condition 1 only) were also included. Because the data for this analysis were not normally distributed, mean test times could not be used. Therefore, the median test times of the last 3 attempts of the first 5 runs during the bead-stringing task were determined for each subject. The results are shown in Figure 2 and Figure s1 in the supplemental material (available at http://jcrsjournal.org), which show that the subjects with stereovision performed better than those in the other 2 groups did ($P \leq 0.014$). Furthermore, no differences between the artificially stereo-deficient and actually stereo-deficient groups were found ($P = 0.351$).

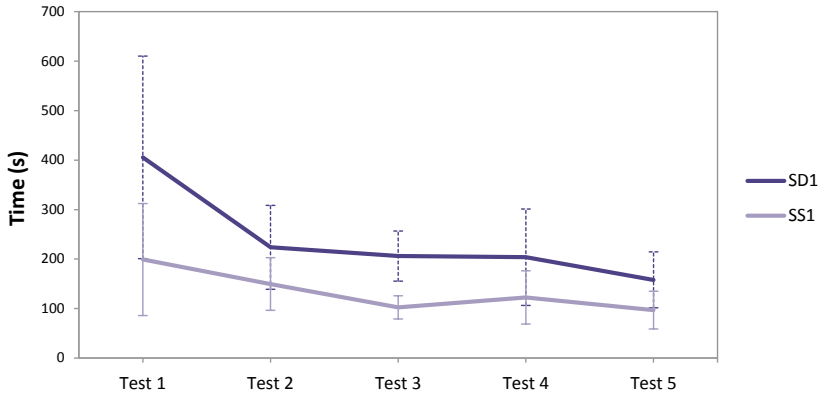


FIGURE 1. *A comparison of the test times on the first 5 runs of the bead stringing task of the stereo-sufficient subjects (SS1, n = 9) and the stereo-deficient subjects (SD1, n = 7). Group means (± standard deviation) are shown. It can be seen that the stereo-sufficient subjects executed the task faster.*

To evaluate microsurgical performance on the bead-stringing task across the different viewing conditions without having to account for intersubject variability, a within-group analysis was performed. Because the data for the within-group analysis were not normally distributed, the median test times under both viewing conditions were used once again.

The graphs in Figure 3 plot the median test times and the test times at the switch of viewing conditions for the 4 different groups of stereo-sufficient subjects used in the bead-stringing task (groups SS1 through SS4, see Table 1). For the subjects in test category SS1, the switch from stereoscopic to binocular viewing resulted in a statistically significant increase in test time (P = 0.008). For the subjects in test category SS2, the median test times were shorter under the stereoscopic condition than under the binocular condition (*P* = 0.008). The median test times of the subjects in category SS3 were shorter under the binocular condition than under the monocular condition (*P* = 0.025).With regard to the subjects in category SS4, no differences between their test times under binocular and monocular conditions were found (*P* ≥ 0.128). Additionally, the results of the slowest and fastest test times for the 4 different groups of stereo-sufficient subjects are shown in Figure s2 in the supplemental material (available at http://jcrsjournal.org).

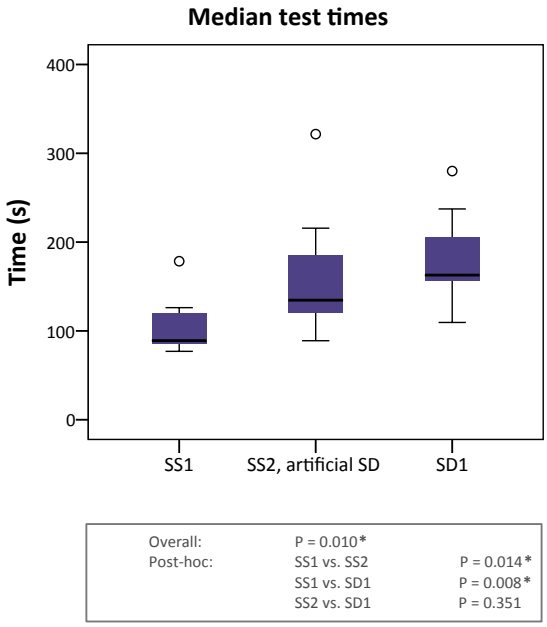


FIGURE 2. *Bead stringing task performance on the first 5 runs of the stereo-sufficient (SS1, n = 9), artificial stereo-deficient (SS2, n = 9), and stereo-deficient (SD1, n = 7) subjects. Comparisons of the median test times are shown. Outliers are indicated with "o". The stereo-sufficient subjects performed the task faster than subjects in the artificial stereo-deficient and actual stereo-deficient groups.*

The possible existence of differences in accuracy of performance between the stereo-sufficient (SS1 through SS4) and stereo-deficient (SD1) subjects was also examined. In Figure 4, test time is plotted as a function of the number of errors. The overall correlation is displayed by a dotted line, which indicates an association between more errors and a longer test time (r_s = 0.27, P < 0.001). This correlation was stronger for the stereo-deficient subjects (SD1, r_s = 0.42, P < 0.001) than for the stereo-sufficient subjects (SS1 through SS4, r_s = 0.22, P < 0.001). However, the difference between the correlations of these groups was not statistically significant ($z_{difference}$ = −1.92, P = 0.055).

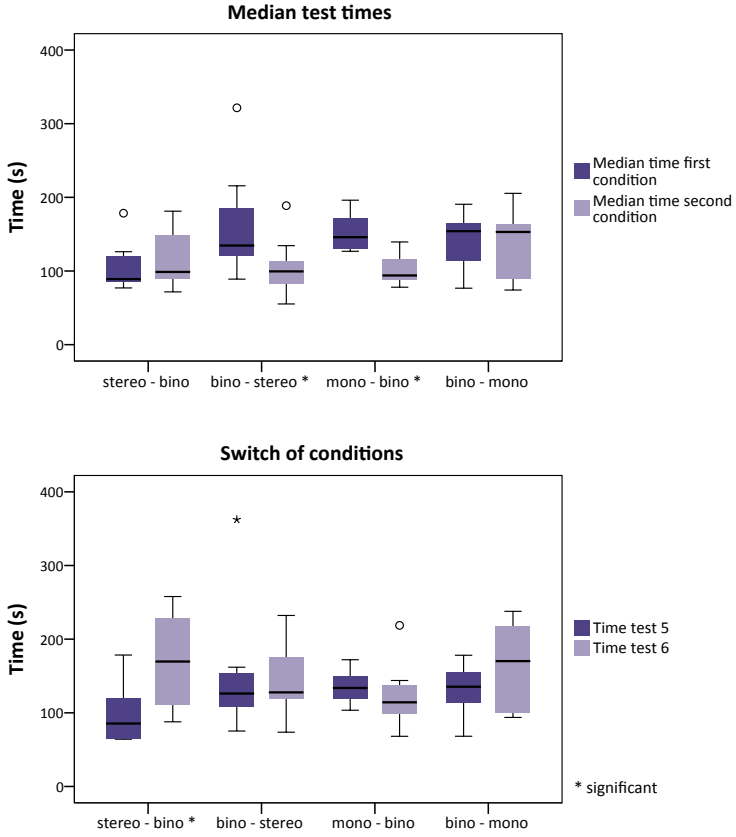


FIGURE 3. *Outcomes of the within-group analysis from the bead stringing task with artificial conditions. The 4 testing categories of stereo-sufficient subjects are displayed as: stereo-bino (SS1, n = 9), bino-stereo (SS2, n = 9), mono-bino (SS3, n = 8), and bino-mono (SS4, n = 7). Outliers are indicated with o and extreme outliers with an asterisk (*). From these plots it is apparent that in general subjects performed better in the stereo condition compared to the binocular condition. The binocular view is more advantageous in comparison with monocular view when the subjects started with monocular conditions.*

Finally, performance of the simulator task by the stereo-sufficient (SS5) subjects was compared with that by the stereo-deficient (SD2) subjects. Figure 5 shows that the median test times did not differ between the groups (P = 0.073). However, the trend in the results suggests faster performance by the stereo-sufficient subjects. Furthermore, the slowest test times (supplemental material and Figure s3, available at http://jcrsjournal.org) of the stereo-deficient (SD2) subjects were statistically significantly longer than those of the stereo-sufficient (SS5) subjects (P = 0.015).

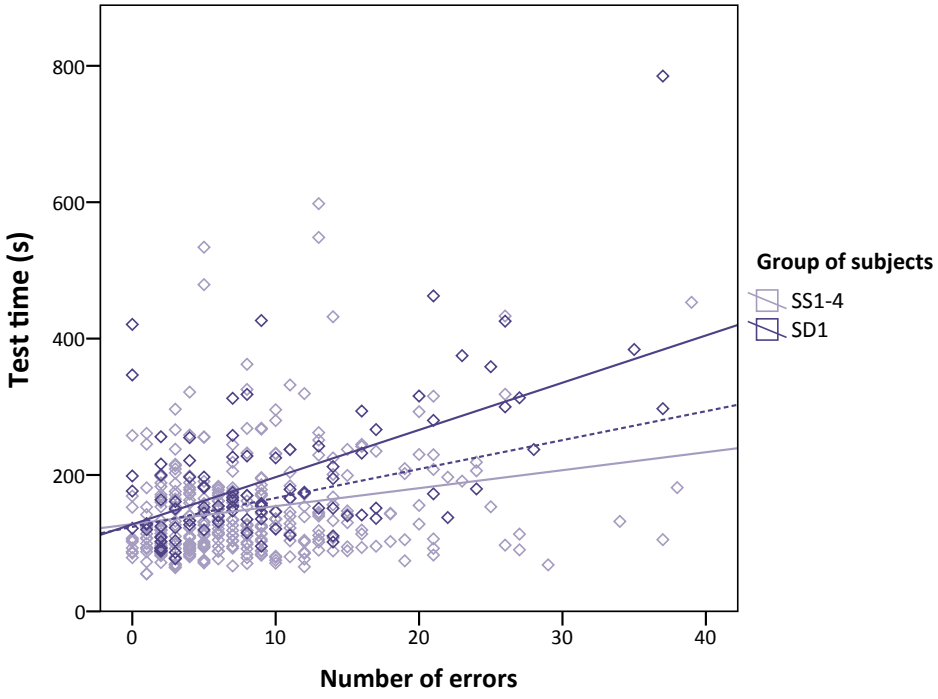


FIGURE 4. *The correlation between test time and number of errors on the bead stringing task. For each subject, the score for each test is shown, providing 10 squares per subject. Errors were scored as 1 point per error. The dotted line indicates the correlation between the increase in performance accuracy and faster test times for both groups together. The black line indicates the correlation for the stereo-deficient subjects (SD1, n = 7) and the gray line the correlation for the stereo-sufficient subjects (SS1-4, n = 33). There is no significant difference between correlations of both groups.*

9.4 Discussion

The ability to combine detailed visual information with fine manual dexterity is considered very important for performing ophthalmic microsurgery. In this study we found a performance benefit of stereoscopic depth perception for tasks executed under an operating microscope and a cataract surgery simulator. The stereo-sufficient subjects performed both tasks faster than the stereo-deficient subjects or artificially stereo-deficient subjects did. Additionally, the stereo-sufficient subjects were faster at executing a bead-stringing task under stereoscopic viewing than were subjects who could not make use of their stereovision (ie, those who were rendered artificially stereo-deficient). Therefore, we conclude that stereovision is advantageous for performing certain tasks under an operating microscope and in a cataract surgery simulator.

Both tasks were also performed by stereo-deficient subjects. We had expected that subjects with a long-term deficiency in stereopsis would have somehow adapted to this situation and developed compensatory strategies. Contrary to our expectation, we found no differences in performance of the bead-stringing task between the artificially stereo-deficient subjects and the actually stereo-deficient subjects. A trend in the results even suggests a slightly faster performance by the artificially stereo-deficient subjects.

Furthermore, we found no differences between stereo-deficient and stereo-sufficient subjects from the standpoint of the correlation between their test time and number of errors on the bead-stringing task. The overall correlation showed that more errors are associated with a longer test time, and in the case of the stereo-deficient deficient subjects, this correlation was somewhat stronger but not statistically significantly different from that in the case of the stereo-sufficient subjects. This finding indicates that stereo-deficient subjects show similar learning curves and that stereo deficiency would not necessarily result in an inability to perform surgical tasks properly. The resemblance in learning curves can also be seen in Figure 1, which shows that the stereo-sufficient subjects performed the task significantly faster. However, the learning curves of both groups (the stereo-sufficient and stereo-deficient subjects) appear similar, which suggests that after prolonged practice, test times for stereo-sufficient and stereo-deficient subjects could become equal. Because we tested only initial performance and did not repeat the tasks after prolonged practice, it is possible that stereo-deficient subjects could eventually achieve performance equal to that of stereo-sufficient subjects.

To our knowledge, this is the first study to compare within-subject microsurgical performance by stereo-sufficient and artificially stereo-deficient subjects with intact stereopsis. Such a within-subject design allowed us to compare the influence of stereopsis on performance of a microsurgical task without having to also account for intersubject variability. A limitation of our study is that we investigated performance on only 1 mockup suturing task (bead-stringing) under a microscope and only 1 simulator task, whereas many more skills are required in ophthalmic surgery. Nevertheless, our finding that stereoscopy does affect execution of the tasks examined suggests that it may also contribute to the overall performance of a surgeon. However, we have to consider the possibility that after prolonged practice, stereo-deficient subjects could still achieve sufficient surgical skills.

Reduced stereovision has previously been associated with poorer performance on bead-stringing tasks without the use of a microscope (O'Connor et al., 2010, Sheedy et al., 1986). The role of stereovision in performance of microsurgery with a microscope was first examined by Grober et al. (2003). Their subjects, all of whom were surgical residents with normal stereoscopic vision, performed a suturing task under normal and monocular conditions. No relationship between the presence of stereovision and microsurgical performance was found. Perhaps the global rating scale used to assess microsurgical performance was not sensitive enough to uncover differences in performance (Martin et al., 1997).

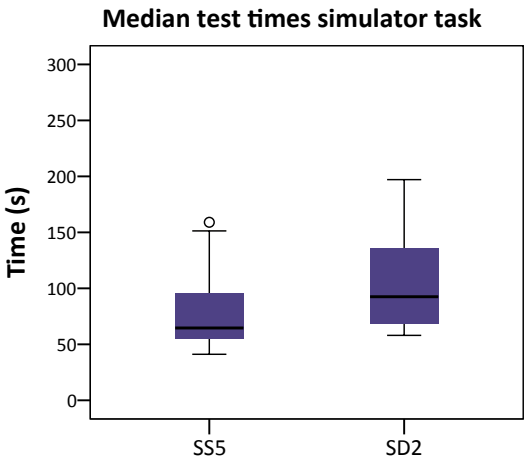


FIGURE 5. *Test times from the simulator task with stereo-sufficient subjects (SS5, n = 28) and stereo-deficient subjects (SD2, n = 9). Outliers are indicated with "o". The Figure shows that there is a trend for median test times in stereo-sufficient subjects to be faster.*

Previous studies did identify an advantage of stereoscopic vision for tasks performed under a cataract surgery simulator. Sachdeva and Traboulsi (2011) found that subjects with a lifelong deficiency or absence of stereovision performed consistently worse than controls with normal stereoacuity did. As in our study, the subjects had no previous surgical experience. In the simulator study of Selvander and Åsman (2011), the subjects were medical students who performed 3 different tasks. Performance was compared among subjects with different levels of stereoacuity. Although the number of subjects with reduced stereoacuity was relatively small (with 3 subjects at 480 arcsec and 5 subjects at >480 arcsec), stereoacuity was correlated with performance on 2 of the 3 simulator tasks. This correlation is in line with our finding that stereoscopic vision is advantageous for performance of tasks on a cataract surgery simulator.

Training involving the use of a cataract surgery simulator is increasingly being implemented in ophthalmology residency programs (McCannel et al., 2013, Saleh et al., 2013). The importance of simulator training is supported by a study in which such training was found to improve the surgical skills of ophthalmology residents (McCannel et al., 2013). Because stereoscopic vision provides a performance advantage during use of a cataract surgery simulator, training outcomes may give some indication of the stereoacuity-dependent surgical competence of the residents. Moreover, because the ability to combine detailed visual information with fine manual dexterity is considered very important for ophthalmology residents, surgery simulators might be envisaged as playing a role in screening residents for admission to ophthalmology resident programs. Further work is required to determine the relationship between initial performance on a simulator as a resident and subsequent performance as a cataract surgeon.

The question whether stereoacuity requirements for ophthalmology resident programs can be justified was recently discussed (Wong et al., 2010).[8] No studies that can provide a direct answer are available. A large retrospective survey on the surgical competency of ophthalmology residents found that 9% had problems developing surgical skills of sufficient quality as judged by their program directors (Binenbaum and Volpe, 2006). In only 3% of these residents were (unspecified) visual problems present as well. The residents with surgical skill problems more often had problems such as poor hand–eye coordination (24%), poor intraoperative judgment (22%), tremor (14%), questionable behavior (11%), and inappropriate reaction to stress in the operating room (10%).

To summarize, the relevance of screening for stereovision for admission to ophthalmology resident programs is not proven, and many additional factors may influence

surgical performance. This study shows that stereoscopic depth perception is beneficial for the initial performance of tasks executed under an operating microscope and on a cataract surgery simulator. Therefore, it is likely that stereovision is also advantageous when learning to perform or actually using surgical skills. Importantly, our study does not show that stereo deficiency would necessarily result in an inability to perform such tasks properly. Hence, in our view, in light of all the possible issues that may hamper the acquisition of surgical skills by ophthalmology residents, it is not evident that stereovision should be judged more stringently than other traits.

Acknowledgements

Drs. Nibourg and Wanders performed this study as part of the curriculum for the master of Medicine at the University of Groningen. The University of Groningen had no role in the design, conduct, analysis, or publication of this research. The authors want to thank students of the University of Groningen who participated in this study and Remco Renken for providing helpful comments made during the design of the experiments.

References

Bach M., 2007. The Freiburg Visual Acuity Test-variability unchanged by post-hoc re-analysis. Graefes Arch. Clin. Exp. Ophthalmol. 245, 965-971.

Binenbaum G., Volpe N.J., 2006. Ophthalmology resident surgical competency: a national survey. Ophthalmology 113, 1237-1244.

Cagenello R., Arditi A., Halpern D.L., 1993. Binocular enhancement of visual acuity. J. Opt. Soc. Am. A Opt. Image Sci. Vis. 10, 1841-1848.

Donzis P.B., Rappazzo J.A., Burde R.M., Gordon M., 1983. Effect of binocular variations of Snellen's visual acuity on Titmus stereoacuity. Arch. Ophthalmol. 101, 930-932.

Goodwin R.T., Romano P.E., 1985. Stereoacuity degradation by experimental and real monocular and binocular amblyopia. Invest. Ophthalmol. Vis. Sci. 26, 917-923.

Grober E.D., Hamstra S.J., Wanzel K.R., Reznick R.K., Matsumoto E.D., Sidhu R.S., Jarvi K.A., 2003. Validation of novel and objective measures of microsurgical skill: Hand-motion analysis and stereoscopic visual acuity. Microsurgery 23, 317-322.

Hoerenz P., 1980. The operating microscope. I. Optical principles, illumination systems, and support systems. J. Microsurg. 1, 364-369.

Martin J.A., Regehr G., Reznick R., MacRae H., Murnaghan J., Hutchison C., Brown M., 1997. Objective structured assessment of technical skill (OSATS) for surgical residents. Br. J. Surg. 84, 273-278.

McCannel C.A., Reed D.C., Goldman D.R., 2013. Ophthalmic surgery simulator training improves resident performance of capsulorhexis in the operating room. Ophthalmology 120, 2456-2461.

Nylen C.O., 1972. The otomicroscope and microsurgery 1921-1971. Acta Otolaryngol. 73, 453-454.

O'Connor A.R., Birch E.E., Anderson S., Draper H., FSOS Research Group, 2010. The functional significance of stereopsis. Invest. Ophthalmol. Vis. Sci. 51, 2019-2023.

Okuda F.C., Apt L., Wanter B.S., 1977. Evaluation of the TNO-random-dot stereogram test. Am. Orthopt. J. 27, 124-130.

Sachdeva R., Traboulsi E.I., 2011. Performance of Patients With Deficient Stereoacuity on the EYESi Microsurgical Simulator. Am. J. Ophthalmol. 151, 427-433.

Saleh G.M., Lamparter J., Sullivan P.M., O'Sullivan F., Hussain B., Athanasiadis I., Litwin A.S., Gillan S.N., 2013. The international forum of ophthalmic simulation: developing a virtual reality training curriculum for ophthalmology. Br. J. Ophthalmol. 97, 789-792.

Selvander M., Asman P., 2011. Stereoacuity and intraocular surgical skill: Effect of stereoacuity level on virtual reality intraocular surgical performance. J. Cataract Refract. Surg. 37, 2188-2193.

Sheedy J.E., Bailey I.L., Buri M., Bass E., 1986. Binocular vs. monocular task performance. Am. J. Optom. Physiol. Opt. 63, 839-846.

Wong N.W., Stokes J., Foss A.J., McGraw P.V., 2010. Should there be a visual standard for ophthalmologists and other surgeons? Postgrad. Med. J. 86, 354-358.

Other cited material

A. Stilma JS, Tan HS, Bijlsma WR. Modernisering Opleiding Oogheelkunde Nederland. 2008
B. Elliott A. Is stereopsis essential to be a competent ophthalmic surgeon? The Royal college of Ophthalmologists - Vision Standards Sub-Committee 2008; September: http://www.rcophth.ac.uk/core/core_picker/download.asp?id=351

Chapter 10

General discussion and future perspectives

10.1 Introduction

This chapter discusses our findings and puts them into the perspective of the current knowledge as described in literature. Additionally, suggestions for further research are made. The main theme of the thesis is cataract surgery and possible improvements in that field. One important topic is capsular opacification (CO). The prevention of capsular opacification has been studied by many researchers, as indicated in Chapter 2 (Nibourg et al., 2015b). Up until now, a proper therapy has not yet been found. The studies described in this thesis intend to examine new methods for the prevention of CO by influencing biological pathways involved in CO formation. Furthermore, in this thesis, we studied the best medium for storage of porcine eyes. Porcine eyes are frequently used in eye surgery training and also in many of our experiments regarding CO. They may be obtained fresh from the slaughterhouse, but it is not always possible to use them immediately due to logistical reasons. Therefore, it is important to know optimal storage conditions. Additionally, we studied the influence of stereopsis on surgical performance with a surgical microscope which may be of interest for the selection and training of future cataract surgeons.

10.2 Prevention of capsular opacification

The prevention of CO is an important field of research in ophthalmology. The main reasons for CO prevention after lens implantation are to maintain a clear visual axis and, for accommodative lenses, to prevent stiffening of the capsular bag. New intraocular lenses, such as injectable accommodative lenses, require an intact capsular bag. Therefore, Nd:YAG laser capsulotomy which destructs the capsular bag is not an option (van Kooten et al., 2006). The importance of the need for prevention of CO was established in Chapter 3 of this thesis, where we found that the formation of CO increases lens stiffness resulting in a reduction of accommodation in lenses designed to accommodate (Nibourg et al., 2015a).

Relation of capsular opacification and lens stiffness

In previous studies of accommodative lens refilling it was already found that CO formation coincided with a decrease of the accommodative amplitude (Koopmans et al., 2006,

Koopmans et al., 2014). However, the combination of a measurement of mechanical lens properties with microscopic imaging of CO has not been performed before. In Chapter 3 we studied mechanical lens properties using a low load compression tester (LLCT). This machine measures the viscoelasticity of the lens by a stress relaxation experiment and is able to determine lens stiffness (Sharma et al., 2011). After a measurement of lens stiffness with the LLCT, the lens can be cultured enabling the development of CO after which the stiffness can be measured again. This helped us to determine the relation between CO formation and the increase in stiffness of the lens capsule.

Prevention of capsular opacification in accommodative lens refilling

Most of the techniques for CO prevention which have been described previously do not specifically aim for prevention of CO in accommodative lens refilling. In Chapter 4 we examined the effect of silicone wettability on the development of CO and lens stiffness. The ideal lens refilling material would have to meet the following characteristics: a refractive index comparable to that of the natural human lens, elastic properties which enable accommodation, and the material would have to counteract the formation of CO (Sharma et al., 2011, van Kooten et al., 2006, Wong et al., 2007). The silicone materials in Chapter 4 have the potential to meet all these characteristics. In that study we examined silicone polymers from two ends of the wettability spectrum. We compared a hydrophobic silicone (silicone ter-polymer) and a hydrophilic silicone (PEG-modified PDMS), and we found no relevant differences in CO formation between both types of silicone. This outcome indicates that material wettability alone does not seem to be relevant for future applications of silicones in accommodative lens refilling.

Treatment with actinomycin D to prevent capsular opacification

We studied the prevention of CO formation by killing the lens epithelial cells (LECs) using a treatment of the lens capsule with the cytotoxic agent actinomycin D in Chapter 3. This agent was already used in other studies for CO prevention, but has proven not to be suitable for use in an open lens capsule *in vivo* because diffusion to other ocular structures in the anterior chamber causes damage (Koopmans et al., 2011, Sternberg et al., 2010, van Kooten et al., 2006). Since in our lens culture model we aimed to compare stiffness of lenses with CO to lenses without CO, we did not have to worry about damage to other ocular tissues. In that study we administered high doses of actinomycin D (50 µg/mL for 1 hour) to the lens capsule of a group of lenses. Nevertheless, even in these lenses viable LECs were detected in the

equatorial regions of the lens capsule (Chapter 3, Figure 4), indicating that the LECs truly are robust. This is in line with the results of other studies, where a treatment with actinomycin D alone provided a decrease yet did not prevent CO formation in rabbit lenses (Koopmans et al., 2011). Only a combination therapy of actinomycin D with methotrexate showed full prevention of CO formation in some rabbits (Sternberg et al., 2010).

Furthermore, we examined the use of actinomycin D dissolved in hyaluronan (10 µg/mL) in Chapter 5. Porcine lens capsules were refilled with hyaluronan containing actinomycin D and showed full CO prevention after three weeks of culturing. The actinomycin was dissolved in hyaluronan, providing a sustained drug-release system to the LECs.

Interference with cellular pathways to prevent capsular opacification

In recent years, many growth factors and signaling molecules have been indentified to be involved in the development of CO (Chapter 2). The growth factor TGF-β has an important role in the onset of CO formation by initiating LEC transdifferentiation (de Iongh et al., 2005, Eldred et al., 2011, Zheng et al., 2012). TGF-β is activated in reaction to tissue trauma and subsequent signaling in the lens is initiated through Smad dependent and Smad independent signaling pathways {{294 Shi,Y. 2003; 145 de Iongh,R.U. 2005}}. Next to TGF-β signaling, also other signaling pathways were found to be related to CO formation (Chapter 2, Figure 4) (Iyengar et al., 2009, Lovicu and McAvoy, 2001). Our studies provided evidence for the involvement of CD44/hyaluronan and integrin signaling pathways in the formation of CO (Chapters 5, 6, and 7).

Hyaluronan and the CD44 receptor were found to be present in human anterior capsules obtained during cataract surgery (Acharya et al., 2008, Nishi et al., 1997a, Saika et al., 1998). The study in Chapter 5 shows that hyaluronan has a dose-dependent effect on LEC morphology. However, we did not find such a clear concentration-dependent effect of hyaluronan on LECs as in the study from Chandler et al. (2012). They treated canine LECs with different concentrations of hyaluronan which showed a concentration-dependent increase in CD44 receptor expression and an increased LEC migration (Chandler et al., 2012). The differences between that study compared to ours may be due to the variation in salt compositions between the hyaluronan solutions, as osmolarity can also influence LEC morphology (Duncan et al., 2007, Rekas et al., 2013).

Interestingly, hyaluronan is a component of the viscoelastics used during current cataract surgery to maintain the anterior chamber of the eye. Therefore, it is important to unravel the effects of hyaluronan on the formation of CO.

The studies described in Chapters 6 and 7 provide evidence for the involvement of integrin signaling in CO formation. Integrins are receptors involved in cell – extracellular matrix (ECM) interactions and were found to be present in LECs (McLean et al., 2005, Zhang et al., 2000). Recently, changes in integrin – ECM interactions have been related to the transdifferentiation of LECs (Mamuya et al., 2014, Worthington et al., 2011). Furthermore, it was found that integrin signaling can activate TGF-β (Dawes et al., 2007, Worthington et al., 2011). Our studies indicate that integrin signaling can be influenced by presenting different ECM peptides to the LECs resulting in changes in LEC transdifferentiation. The peptides used in our studies were derived from the ECM components laminin, fibronectin, and collagen IV, which have all been related to CO formation in the lens (Linnola et al., 2000, Olivero and Furcht, 1993). Up until now, only a few studies have analyzed the effect of specific ECM peptides on integrin signaling. Nishi et al. (1997b) studied the effect of the fibronectin-derived RGD-peptide for CO prevention and showed a slight inhibition of LEC migration, but this was not found to be significant. The combination therapy of RGD-peptide with the chemical agent EDTA (ethylenediamine tetraacetic acid), however, provided significant inhibition of CO formation (Inan et al., 2001, Nishi et al., 1997b). In our studies we did not combine the administration of ECM peptides with other chemical therapy, but we discovered that a combination of two or more peptides contributes to a large effect on LEC morphology. This again indicates that combination therapies are more effective than monotherapy.

Nanomaterials for the prevention of capsular opacification

In Chapters 6 and 7 we studied the influence of nanofiber-based hydrogels (nanogels) on cell processes in the LECs by presenting ECM peptide moieties to the cells. Hydrogels and nanogels are preferred for many medical applications due to their permeable and flexible nature, which makes them able to resemble natural tissues (Chapter 2, Figure 6) (Etheridge et al., 2013, Goldberg et al., 2007). In ophthalmology, nanomaterials have been examined for different applications (Ellis-Behnke and Jonas, 2011, Ionescu et al., 2011, Kaiser et al., 2013, Pritchard et al., 2010), however, only few studies reported on nanomaterials for CO prevention by using them as a drug-delivery system through intraocular lens (IOL) coatings or sustained release by encapsulation (Guha et al., 2013, Huang et al., 2013, Zhang et al., 2013). In our studies, nanogels were used as a delivery system. We presented different combinations of ECM peptides to the LECs by refilling the lens capsule with a nanogel in combination with covalently bound peptides. With this method we were able test various

combinations of ECM peptides in order to influence LEC behavior. Another advantage of using nanogels to refill the lens capsule is that peptides are directly presented to the surface of the LECs, which enabled us to directly influence LEC behavior through integrin signaling. The permeable and flexible nature of nanogels and the potential to link peptides and incorporate other agents, makes nanogels and nanomaterials in general promising materials for studying their role in CO modulation and possibly prevention. Our data in Chapters 6 and 7 demonstrate that nanogels can influence the LEC response and that small changes in peptide ratios can have large effects on the cellular processes in the LECs.

10.3 Improvement of technical aspects in cataract surgery

We studied the extent in which stereoscopic vision is related to microsurgical task performance in Chapter 9. Generally, there is the belief that there is a relation between a lack of stereovision and poor surgical skills in residents.[A] However, there are no studies which provide direct evidence for this belief. A paper by Wong et al. (Wong et al., 2010) discusses the question whether visual standards can be justified for admission to ophthalmology resident programs. A questionnaire was sent to ophthalmologists in the United Kingdom which revealed that 80% of the responders thought there should be visual standards and 93% of the responders wanted to screen for stereovision. Another survey performed in the United States on surgical competency of residents in ophthalmology reported 9% residents with problems in developing surgical skills, but only 3% of these residents had (unspecified) visual problems (Binenbaum and Volpe, 2006). These outcomes show the urge for studies on this subject. In our study, we found a benefit of stereovision for performance with the surgical microscope as well as with a cataract surgery simulator. Other studies on the effect of stereovision on performance with a cataract surgery simulator also showed better performance of subjects with better stereovision (Sachdeva and Traboulsi, 2011, Selvander and Asman, 2011). However, we need to take into account that it is still possible that stereo-deficient residents could end up with sufficient surgical skills after prolonged practice. Taken together, it seems that there is a benefit for stereovision on microsurgical task performance. However, the relevance for screening for stereovision could not be determined, since there are many additional factors which can influence surgical skills and it has not yet been determined if there is an actual disadvantage of stereo-deficiency for surgical task performance.

Another factor that is essential for the development of surgical techniques is the availability of a proper wet laboratory (wet lab) (Henderson et al., 2009). In the University Medical Center Groningen we are fortunate to have good wet lab facilities. In a wet lab, fresh animal eyes are used to train eye surgery and experimental eye surgery procedures. However, post mortem changes in animal eyes may cause opacification of the cornea which reduces visibility of the eye structures. With the study described in Chapter 8, we wanted to maintain the optical clarity of the corneas from the porcine eyes used in our wet lab. We found that preservation of the porcine eyes in tap water (mineral content 1.79 mmol/L) provided the least corneal swelling, resulting in the best corneal transparency (Jester et al., 2001, Maurice, 1957). These outcomes are interesting because tap water is easily available in all wet labs. Furthermore, there was no need to use the more expensive solutions which are normally used during cataract surgery (Nibourg and Koopmans, 2014). So far, there are no studies which have evaluated the use of tap water for corneal preservation and some research has been carried out using other corneal preservation methods. Solutions containing e.g. saline, Ringer lactate, glucose-phosphate, methylparaben, propylparaben, glycerol, and dextran were examined (Auffarth et al., 1996, Igarashi et al., 1993, Liu et al., 2001, Swinger and Kornmehl, 1985). However, none of these studies evaluated the wide range of preservation methods and long preservation times as in our study, which makes comparisons difficult. In our study we showed that porcine eyes can be well preserved in tap water for a period up to 50 hours, making this a good method for porcine eye preservation in wet labs.

Experimental models in cataract surgery research

Appropriate use of experimental models is highly important for research in cataract surgery, as shown in Chapters 3, 4, 5, 6, and 7. Many experimental models have been described for testing methods for improvement of surgical techniques or prevention of CO (Ashwin et al., 2009, Wormstone and Eldred, 2015), and all methods have their own advantages and disadvantages. For the experimental studies in this thesis, we used a porcine eye model. This model has been used more often, but also other models are commonly described (Lee et al., 2008, Mitani et al., 2014, Wormstone and Eldred, 2015). The reason for using porcine eyes in our studies was based on their adequate size for performing accommodative lens refilling surgeries and cataract surgery in general (Henderson et al., 2009, Nibourg and Koopmans, 2014). Furthermore, porcine eyes were easily available in our facility and the extracted lenses are well maintained in organ cultures. The antibodies

which we wanted to use for immunocytochemistry showed affinity for pig tissues, thereby providing opportunities for analysis of the cellular processes in the LECs (Chapters 5, 6, and 7). Next to the porcine eye model, other experimental models would have been suitable to achieve our objectives. First, primary cell cultures and continuous cell lines could be used to examine the underlying biological processes of CO formation and the effect of nanogels on these processes. A major advantage of cell culturing is the extent to which various factors can be regulated. However, cell lines do not show variation in cell types and are therefore not comparable to the human situation (Nibourg et al., 2015b). For the evaluation of our surgical model and the refilling techniques *ex vivo*, human cadaveric eyes could have been an option. However, their limited availability, also with regard to the relatively long post-mortem time, and the experimental setting of our experiments made that we decided to start evaluating our materials in porcine eyes. The next step in the development of new materials and techniques could be *in vivo* experiments. Appropriate animals are rabbits or monkeys (Behar-Cohen et al., 1995, Koopmans et al., 2006, Koopmans et al., 2011, Nishi and Nishi, 1998), as further discussed in the future perspectives of this thesis (section 10.4).

10.4 Future perspectives

The findings reported in this thesis make several contributions to the current literature. They can be used to improve techniques in cataract surgery and methods for CO prevention.

For the implementation of accommodative lens refilling further *in vivo* experiments are required. The lens refilling technique has already been examined in rabbit and monkey models and it has been shown that this technique can restore accommodation (Behar-Cohen et al., 1995, Koopmans et al., 2006, Koopmans et al., 2011, Nishi and Nishi, 1998). The next steps will be to evaluate the refractive power in refilled lenses and to further explore the possibilities for CO prevention *in vivo* (Koopmans et al., 2004, van Kooten et al., 2006).

Further research in the prevention of CO should focus on determination of the biological pathways that cause CO formation. It is important to understand the specific steps in the processes of epithelial to mesenchymal transition (EMT) in the LECs. This knowledge can then be used provide better targets for therapies in CO prevention.

Most studies only test one specific method for CO prevention, whilst it already has been hypothesized that probably combination therapies are needed to achieve total CO prevention (Nibourg et al., 2015b). This is also evident from the experimental data presented in this thesis. In Chapter 5 the combination hyaluronan and actinomycin D resulted in CO prevention and in Chapter 6 and 7 combinations of ECM peptides provided a larger effect on the LEC response compared to monotherapy. The schematic overview of interactions between TGF-β signaling and other signaling pathways in Figure 4 from Chapter 2 demonstrates the complexity of the process of EMT in the lens and also indicates that probable a combination of more than one target will be needed to totally prevent CO. In our studies we examined only the integrin and hyaluronan/CD44 signaling pathways. However, the complexity of the cellular processes shows that it is probably necessary to also target other signaling pathways in order to achieve a more complete CO prevention. The challenge is to find suitable combinations of biological targets. In this, nanomaterials can play an important role because of their resemblance to natural tissue and their possibilities to be used as a delivery system for other agents. Therefore, we believe that in future methods nanomaterials targeting biological pathways are a promising avenue towards CO prevention.

It is not yet known whether a complete CO prevention will be necessary in future techniques for cataract surgery. Recent developments in research to lens regeneration describe methods where a clear lens can be formed by regrowing of lens fibers (Gwon and Gruber, 2010). In this technique a scaffold is used to mimic the natural environment of the LECs in order to induce lens regeneration. Next to this, it must be taken into account that full CO prevention could entail other difficulties. There is evidence that an absence of LECs may cause problems with fixation of the IOL in the capsular bag (Spalton et al., 2014). Normally, the IOL is secured in the capsular bag by LEC growth and fibrosis around the haptics of the IOL. Consequences of an insufficient IOL fixation could be displacement or movement of the IOL. Additionally, effects of an absence of LEC on the capsular bag are unknown. It might be that some remaining LEC are required to retain the integrity of capsular bag. Thus, further studies regarding the role of LEC on lens capsule integrity would be worthwhile.

10.5 Conclusion

The studies in this thesis contribute to the knowledge of the biological processes underlying the formation of capsular opacification in the lens and provide insights into possible methods for the prevention of capsular opacification and the improvement of surgical techniques in cataract surgery. Considerably more work is needed to unravel the specific processes of epithelial to mesenchymal transition causing capsular opacification. The challenge for next generation cataract surgery is to develop biological targeted interventions which prevent capsular opacification.

References

Acharya P.S., Majumdar S., Jacob M., Hayden J., Mrass P., Weninger W., Assoian R.K., Pure E., 2008. Fibroblast migration is mediated by CD44-dependent TGF beta activation. J. Cell. Sci. 121, 1393-1402.

Ashwin P.T., Shah S., Wolffsohn J.S., 2009. Advances in cataract surgery. Clin. Exp. Optom. 92, 333-342.

Auffarth G.U., Wesendahl T.A., Solomon K.D., Brown S.J., Apple D.J., 1996. A modified preparation technique for closed-system ocular surgery of human eyes obtained postmortem: an improved research and teaching tool. Ophthalmology 103, 977-982.

Behar-Cohen F.F., David T., D'Hermies F., Pouliquen Y.M., Buechler Y., Nova M.P., Houston L.L., Courtois Y., 1995. In vivo inhibition of lens regrowth by fibroblast growth factor 2-saporin. Invest. Ophthalmol. Vis. Sci. 36, 2434-2448.

Binenbaum G., Volpe N.J., 2006. Ophthalmology resident surgical competency: a national survey. Ophthalmology 113, 1237-1244.

Chandler H.L., Haeussler D.J.,Jr, Gemensky-Metzler A.J., Wilkie D.A., Lutz E.A., 2012. Induction of posterior capsule opacification by hyaluronic acid in an ex vivo model. Invest. Ophthalmol. Vis. Sci. 53, 1835-1845.

Chen X., Ye S., Xiao W., Wang W., Luo L., Liu Y., 2014. ERK1/2 pathway mediates epithelial-mesenchymal transition by cross-interacting with TGFbeta/Smad and Jagged/Notch signaling pathways in lens epithelial cells. Int. J. Mol. Med. 33, 1664-1670.

Cho H.J., Baek K.E., Saika S., Jeong M.J., Yoo J., 2007. Snail is required for transforming growth factor-beta-induced epithelial-mesenchymal transition by activating PI3 kinase/Akt signal pathway. Biochem. Biophys. Res. Commun. 353, 337-343.

Cho H.J., Yoo J., 2007. Rho activation is required for transforming growth factor-beta-induced epithelial-mesenchymal transition in lens epithelial cells. Cell Biol. Int. 31, 1225-1230.

Dawes L.J., Elliott R.M., Reddan J.R., Wormstone Y.M., Wormstone I.M., 2007. Oligonucleotide microarray analysis of human lens epithelial cells: TGFbeta regulated gene expression. Mol. Vis. 13, 1181-1197.

de Iongh R.U., Wederell E., Lovicu F.J., McAvoy J.W., 2005. Transforming growth factor-beta-induced epithelial-mesenchymal transition in the lens: a model for cataract formation. Cells Tissues Organs 179, 43-55.

Duncan G., Wang L., Neilson G.J., Wormstone I.M., 2007. Lens cell survival after exposure to stress in the closed capsular bag. Invest. Ophthalmol. Vis. Sci. 48, 2701-2707.

Eldred J.A., Dawes L.J., Wormstone I.M., 2011. The lens as a model for fibrotic disease. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 366, 1301-1319.

Ellis-Behnke R., Jonas J.B., 2011. Redefining tissue engineering for nanomedicine in ophthalmology. Acta Ophthalmol. 89, e108-114.

Erpelding T.N., Hollman K.W., O'Donnell M., 2007. Mapping age-related elasticity changes in porcine lenses using bubble-based acoustic radiation force. Exp. Eye Res. 84, 332-341.

Etheridge M.L., Campbell S.A., Erdman A.G., Haynes C.L., Wolf S.M., McCullough J., 2013. The big picture on nanomedicine: the state of investigational and approved nanomedicine products. Nanomedicine 9, 1-14.

Fisher R.F., 1977. The force of contraction of the human ciliary muscle during accommodation. J. Physiol. 270, 51-74.

Goldberg M., Langer R., Jia X., 2007. Nanostructured materials for applications in drug delivery and tissue engineering. J. Biomater. Sci. Polym. Ed. 18, 241-268.

Guha R., Chowdhury S., Palui H., Mishra A., Basak S., Mandal T.K., Hazra S., Konar A., 2013. Doxorubicin-loaded MePEG-PCL nanoparticles for prevention of posterior capsular opacification. Nanomedicine (Lond) 8, 1415-1428.

Gwon A., Gruber L., 2010. Engineering the crystalline lens with a biodegradable or non-degradable scaffold. Exp. Eye Res. 91, 220-228.

Henderson B.A., Grimes K.J., Fintelmann R.E., Oetting T.A., 2009. Stepwise approach to establishing an ophthalmology wet laboratory. J. Cataract Refract. Surg. 35, 1121-1128.

Hermans E.A., Pouwels P.J., Dubbelman M., Kuijer J.P., van der Heijde R.G., Heethaar R.M., 2009. Constant volume of the human lens and decrease in surface area of the capsular bag during accommodation: an MRI and Scheimpflug study. Invest. Ophthalmol. Vis. Sci. 50, 281-289.

Huang X., Wang Y., Cai J.P., Ma X.Y., Li Y., Cheng J.W., Wei R.L., 2013. Sustained release of 5-fluorouracil from chitosan nanoparticles surface modified intra ocular lens to prevent posterior capsule opacification: an in vitro and in vivo study. J. Ocul. Pharmacol. Ther. 29, 208-215.

Igarashi H., Yoshida A., Tanaka K., 1993. Comparison of pig cornea preservation solutions using quantitative 31P NMR spectroscopy. Magn. Reson. Med. 29, 107-109.

Inan U.U., Ozturk F., Kaynak S., Kurt E., Emiroglu L., Ozer E., Ilker S.S., Guler C., 2001. Prevention of posterior capsule opacification by intraoperative single-dose pharmacologic agents. J. Cataract Refract. Surg. 27, 1079-1087.

Ionescu A.M., Alaminos M., de la Cruz Cardona J., de Dios Garcia-Lopez Duran J., Gonzalez-Andrades M., Ghinea R., Campos A., Hita E., del Mar Perez M., 2011. Investigating a novel nanostructured fibrin-agarose biomaterial for human cornea tissue engineering: rheological properties. J. Mech. Behav. Biomed. Mater. 4, 1963-1973.

Iyengar L., Patkunanathan B., McAvoy J.W., Lovicu F.J., 2009. Growth factors involved in aqueous humour-induced lens cell proliferation. Growth Factors 27, 50-62.

Jester J.V., Ghee Lee Y., Li J., Chakravarti S., Paul J., Petroll W.M., Dwight Cavanagh H., 2001. Measurement of corneal sublayer thickness and transparency in transgenic mice with altered corneal clarity using in vivo confocal microscopy. Vision Res. 41, 1283-1290.

Kaiser J.M., Imai H., Haakenson J.K., Brucklacher R.M., Fox T.E., Shanmugavelandy S.S., Unrath K.A., Pedersen M.M., Dai P., Freeman W.M., Bronson S.K., Gardner T.W., Kester M., 2013. Nanoliposomal minocycline for ocular drug delivery. Nanomedicine 9, 130-140.

Koopmans S.A., Terwee T., Barkhof J., Haitjema H.J., Kooijman A.C., 2003. Polymer refilling of presbyopic human lenses in vitro restores the ability to undergo accommodative changes. Invest. Ophthalmol. Vis. Sci. 44, 250-257.

Koopmans S.A., Terwee T., Glasser A., Wendt M., Vilupuru A.S., van Kooten T.G., Norrby S., Haitjema H.J., Kooijman A.C., 2006. Accommodative lens refilling in rhesus monkeys. Invest. Ophthalmol. Vis. Sci. 47, 2976-2984.

Koopmans S.A., Terwee T., Haitjema H.J., Deuring H., Aarle S., Kooijman A.C., 2004. Relation between injected volume and optical parameters in refilled isolated porcine lenses. Ophthalmic Physiol. Opt. 24, 572-579.

Koopmans S.A., Terwee T., Hanssen A., Martin H., Langner S., Stachs O., van Kooten T.G., 2014. Prevention of capsule opacification after accommodating lens refilling: pilot study of strategies evaluated in a monkey model. J. Cataract Refract. Surg. 40, 1521-1535.

Koopmans S.A., Terwee T., van Kooten T.G., 2011. Prevention of capsular opacification after accommodative lens refilling surgery in rabbits. Biomaterials 32, 5743-5755.

Lee S.J., Bae S., Seomun Y., Son M.J., Joo C.K., 2008. The role of nuclear factor kappa B in lens epithelial cell proliferation using a capsular bag model. Ophthalmic Res. 40, 273-278.

Linnola R.J., Werner L., Pandey S.K., Escobar-Gomez M., Znoiko S.L., Apple D.J., 2000. Adhesion of fibronectin, vitronectin, laminin, and collagen type IV to intraocular lens materials in pseudophakic human autopsy eyes. Part 1: histological sections. J. Cataract Refract. Surg. 26, 1792-1806.

Liu E.S., Eng K.T., Braga-Mele R., 2001. Using medical lubricating jelly in human cadaver eyes to teach ophthalmic surgery. J. Cataract Refract. Surg. 27, 1545-1547.

Lovicu F.J., McAvoy J.W., 2001. FGF-induced lens cell proliferation and differentiation is dependent on MAPK (ERK1/2) signalling. Development 128, 5075-5084.

Mamuya F.A., Wang Y., Roop V.H., Scheiblin D.A., Zajac J.C., Duncan M.K., 2014. The roles of alphaV integrins in lens EMT and posterior capsular opacification. J. Cell. Mol. Med. 18, 656-670.

Maurice D.M., 1957. The structure and transparency of the cornea. J. Physiol. 136, 263-286.

McLean S.M., Mathew M.R., Kelly J.B., Murray S.B., Bennett H.G., Webb L.A., Esakowitz L., McLean J.S., 2005. Detection of integrins in human cataract lens epithelial cells and two mammalian lens epithelial cell lines. Br. J. Ophthalmol. 89, 1506-1509.

Mitani A., Suzuki T., Tasaka Y., Uda T., Hiramatsu Y., Kawasaki S., Ohashi Y., 2014. Evaluation of a new method of irrigation and aspiration for removal of ophthalmic viscoelastic device during cataract surgery in a porcine model. BMC Ophthalmol. 14, 129-2415-14-129.

Nibourg L.M., Koopmans S.A., 2014. Preservation of enucleated porcine eyes for use in a wet laboratory. J. Cataract Refract. Surg. 40, 644-651.

Nibourg L.M., Sharma P.K., van Kooten T.G., Koopmans S.A., 2015a. Changes in lens stiffness due to capsular opacification in accommodative lens refilling. Exp. Eye Res. 134, 148-154.

Nibourg L.M., Gelens E., Kuijer R., Hooymans J.M.M., van Kooten T.G., Koopmans S.A., 2015b. Prevention of posterior capsular opacification. Exp. Eye Res. 136, 100-115.

Nishi O., Nishi K., 1998. Accommodation amplitude after lens refilling with injectable silicone by sealing the capsule with a plug in primates. Arch. Ophthalmol. 116, 1358-1361.

Nishi O., Nishi K., Akaishi T., Shirasawa E., 1997a. Detection of cell adhesion molecules in lens epithelial cells of human cataracts. Invest. Ophthalmol. Vis. Sci. 38, 579-585.

Nishi O., Nishi K., Mano C., Ichihara M., Honda T., Saitoh I., 1997b. Inhibition of migrating lens epithelial cells by blocking the adhesion molecule integrin: a preliminary report. J. Cataract Refract. Surg. 23, 860-865.

Olivero D.K., Furcht L.T., 1993. Type IV collagen, laminin, and fibronectin promote the adhesion and migration of rabbit lens epithelial cells in vitro. Invest. Ophthalmol. Vis. Sci. 34, 2825-2834.

Pritchard C.D., Arner K.M., Langer R.S., Ghosh F.K., 2010. Retinal transplantation using surface modified poly(glycerol-co-sebacic acid) membranes. Biomaterials 31, 7978-7984.

Reilly M.A., Hamilton P.D., Perry G., Ravi N., 2009. Comparison of the behavior of natural and refilled porcine lenses in a robotic lens stretcher. Exp. Eye Res. 88, 483-494.

Rekas M., Klus A., Kosatka M., 2013. Sealed-capsule irrigation with distilled deionized water to prevent posterior capsule opacification--prospective, randomized clinical trial. Curr. Eye Res. 38, 363-370.

Sachdeva R., Traboulsi E.I., 2011. Performance of Patients With Deficient Stereoacuity on the EYESi Microsurgical Simulator. Am. J. Ophthalmol. 151, 427-433.

Saika S., Kawashima Y., Miyamoto T., Okada Y., Tanaka S., Yamanaka O., Ohnishi Y., Ooshima A., Yamanaka A., 1998. Immunolocalization of hyaluronan and CD44 in quiescent and proliferating human lens epithelial cells. J. Cataract Refract. Surg. 24, 1266-1270.

Selvander M., Asman P., 2011. Stereoacuity and intraocular surgical skill: Effect of stereoacuity level on virtual reality intraocular surgical performance. J. Cataract Refract. Surg. 37, 2188-2193.

Sharma P.K., Busscher H.J., Terwee T., Koopmans S.A., van Kooten T.G., 2011. A comparative study on the viscoelastic properties of human and animal lenses. Exp. Eye Res. 93, 681-688.

Shi Y., Massague J., 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113, 685-700.

Spalton D.J., Russell S.L., Evans-Gowing R., Eldred J.A., Wormstone I.M., 2014. Effect of total lens epithelial cell destruction on intraocular lens fixation in the human capsular bag. J. Cataract Refract. Surg. 40, 306-312.

Sternberg K., Terwee T., Stachs O., Guthoff R., Lobler M., Schmitz K.P., 2010. Drug-induced secondary cataract prevention: experimental ex vivo and in vivo results with disulfiram, methotrexate and actinomycin D. Ophthalmic Res. 44, 225-236.

Strenk S.A., Semmlow J.L., Strenk L.M., Munoz P., Gronlund-Jacob J., DeMarco J.K., 1999. Age-related changes in human ciliary muscle and lens: a magnetic resonance imaging study. Invest. Ophthalmol. Vis. Sci. 40, 1162-1169.

Swinger C.A., Kornmehl E.W., 1985. Dehydration of post-mortem eyes for practice and experimental surgery. Ophthalmic Surg. 16, 182-183.

van Kooten T.G., Koopmans S., Terwee T., Norrby S., Hooymans J.M., Busscher H.J., 2006. Development of an accommodating intra-ocular lens--in vitro prevention of re-growth of pig and rabbit lens capsule epithelial cells. Biomaterials 27, 5554-5560.

Wong K.H., Koopmans S.A., Terwee T., Kooijman A.C., 2007. Changes in spherical aberration after lens refilling with a silicone oil. Invest. Ophthalmol. Vis. Sci. 48, 1261-1267.

Wong N.W., Stokes J., Foss A.J., McGraw P.V., 2010. Should there be a visual standard for ophthalmologists and other surgeons? Postgrad. Med. J. 86, 354-358.

Wormstone I.M., Eldred J.A., 2015. Experimental models for posterior capsule opacification research. Exp. Eye Res. Epub.

Worthington J.J., Klementowicz J.E., Travis M.A., 2011. TGFbeta: a sleeping giant awoken by integrins. Trends Biochem Sci 36, 47-54.

Yoon S., Aglyamov S., Karpiouk A., Emelianov S., 2013. The mechanical properties of ex vivo bovine and porcine crystalline lenses: age-related changes and location-dependent variations. Ultrasound Med. Biol. 39, 1120-1127.

Zhang W., Li X., Ye T., Chen F., Sun X., Kong J., Yang X., Pan W., Li S., 2013. Design, characterization, and in vitro cellular inhibition and uptake of optimized genistein-loaded NLC for the prevention of posterior capsular opacification using response surface methodology. Int. J. Pharm. 454, 354-366.

Zhang X.H., Ji J., Zhang H., Tang X., Sun H.M., Yuan J.Q., 2000. Detection of integrins in cataract lens epithelial cells. J. Cataract Refract. Surg. 26, 287-291.

Zheng D., Song T., Zhongliu X., Wu M., Liang J., Liu Y., 2012. Downregulation of transforming growth factor-beta type II receptor prohibit epithelial-to-mesenchymal transition in lens epithelium. Mol. Vis. 18, 1238-1246.

Zhong J., Tao A., Xu Z., Jiang H., Shao Y., Zhang H., Liu C., Wang J., 2014. Whole eye axial biometry during accommodation using ultra-long scan depth optical coherence tomography. Am. J. Ophthalmol. 157, 1064-1069.

Other cited material

A. Stilma J.S., Tan H.S., Bijlsma W.R., 2008. Modernisering Opleiding Oogheelkunde Nederland. Available at: http://www.oogheelkunde.org/professionals/opleiding/concilium2/moon-versie-mei-2008. Accessed July 8, 2015

Chapter 11

Summary

Cataract causes a decrease in visual acuity due to opacification of the components of the lens and is treated with replacement of those components by an intraocular lens (IOL) by cataract surgery. Capsular opacification (CO) is a common complication of cataract surgery and involves growth (e.g. proliferation, migration, and transdifferentiation) of lens epithelial cells (LECs), which remained in the lens capsule after cataract surgery. This results in a second decrease in visual acuity. The underlying cellular processes and the possible methods for the prevention of CO are extensively studied, but a proper therapy preventing CO has not yet been found. **Chapter 1** provides an introduction and defines the aims of the thesis. **Chapter 2** describes the current knowledge of CO prevention by providing insights into the underlying biological processes and discusses the various methods which have been studied.

In **Chapter 3** we report the relation between the formation of CO and the increase in lens stiffness. We performed stress relaxation experiments to determine lens stiffness in lenses refilled with a silicone polymer for accommodative lens refilling. We found that lens stiffening coincides with the development of CO. This is an important outcome because increased lens stiffness results in a decrease in accommodative amplitude in accommodative lens refilling and stresses the need for CO prevention for new techniques in cataract surgery.

Chapter 4 describes our study in which different types of silicone polymers were examined for their effect on the lens stiffness and the response of the LECs in accommodative lens refilling. The silicone polymers were chosen from the two ends of the wettability spectrum: a hydrophobic silicone (silicone ter-polymer) and a hydrophilic silicone (PEG-modified PDMS). There were no relevant differences in CO formation between both types of silicone, which indicates that material wettability alone does not seem to be relevant for future applications of silicones in accommodative lens refilling.

Our study in **Chapter 5** was performed to examine the effect of hyaluronan (sodium hyaluronate) on CO formation. Hyaluronan is a component of the viscoelastics which are currently used during cataract surgery and we found that the LEC response can be influenced by different compositions of hyaluronan dissolved in saline and purified water. The addition of the cytotoxic agent actinomycin D provided lenses with a total absence of LECs. Despite the fact that the use of actinomycin D in the lens is possibly dangerous because of side effects to other ocular tissues, we showed that a combination therapy of hyaluronan and actinomycin D resulted in a total absence of LEC.

In Chapter 6 we showed that nanofiber-based hydrogels (nanogels) can influence the LEC response. We compared the results of 14 different combinations of nanogels with attached peptides. The combinations were created by five peptides derived from the extracellular matrix (ECM) components laminin, fibronectin, and collagen IV, which all have been related to the formation of CO via integrin signaling pathways. With this study we provided evidence for the involvement of integrin signaling in CO formation. Furthermore, we were able to decrease the LEC response with specific combinations of peptides, indicating that nanogels are promising materials in research to CO prevention. We explored these outcomes further in Chapter 7, where peptide ratios were optimized for the different nanogels. The study revealed that small changes in peptide ratios can have a large effect on the LECs. Thus, for intergrin signaling ECM peptide concentrations are highly important and further improvement of peptide concentrations in future studies may result in total prevention of CO.

Our experiments in Chapter 8 were performed in order to improve the methods for preservation of porcine eyes for use in a wet laboratory (wet lab). From the 15 methods we tested, preservation in tap water provided the least corneal swelling, which has been correlated to the best corneal transparency. Those are interesting results because tap water is an inexpensive method and easily available in wet labs. Furthermore, we showed that porcine eyes can be well preserved for a period up to 50 hours after extraction. This can make planning of wet lab training and experiments more manageable.

Next to wet lab facilities, also proper surgical training is important for development of surgical techniques. In Chapter 9 we evaluate the importance of stereoscopic vision (stereovision) on surgical performance with the operating microscope. We found a benefit for stereovision for the performance with a surgical microscope and a cataract surgery simulator. However, there are many additional factors which may influence surgical skills and it is unknown if stereo-deficiency will result in an inability to perform surgical tasks.

The overall findings of our studies were discussed in Chapter 10. This chapter puts our results into perspective and provides ideas for future research. We hypothesized that in future methods nanomaterials can be used to target biological pathways in order to prevent CO. Furthermore, we believe that a combination of more than one target is necessary to achieve full prevention of CO.

The studies in this thesis contribute to the knowledge of the underlying biological processes of CO. Insights into possible methods for CO prevention were provided and methods for the improvement of surgical techniques in cataract surgery were presented.

Considerably more work is needed to unravel the specific processes which cause CO in the lens, and the challenge for next generation cataract surgery is to develop biological targeted interventions aimed at the prevention of CO.

Chapter 12

Samenvatting

Staar, ook wel cataract genoemd, veroorzaakt een afname van de gezichtsscherpte als gevolg van lensvertroebeling. De behandeling van staar bestaat uit een staaroperatie waarbij de troebele lens wordt vervangen door een heldere kunstlens. Een bijwerking van de staaroperatie die regelmatig voorkomt is de vorming van nastaar. Nastaar ontstaat als gevolg van groei (proliferatie, migratie en transdifferentiatie) van lensepitheel cellen die na de operatie achterblijven in het lenskapsel. Dit zorgt voor een tweede afname van het gezichtsvermogen van de patiënt. Momenteel wordt nastaar behandeld met een laserbehandeling, maar er wordt ook veel onderzoek verricht naar de onderliggende processen die zich in de cellen afspelen. Als deze processen beïnvloed kunnen worden zouden we zo nastaar kunnen voorkomen. De preventie van nastaar is ook van belang voor de ontwikkeling van nieuwe technieken voor staaroperaties, zoals injecteerbare accommoderende lenzen. Bij deze techniek worden de troebele lensvezels vervangen door een flexibele siliconen gel, zodat accommodatie van de lens weer mogelijk is en er geen leesbril meer nodig is. **Hoofdstuk 1** van dit proefschrift geeft een introductie en behandelt de doelen van onze studies. **Hoofdstuk 2** beschrijft de recente kennis op het gebied van de preventie van nastaar. Hierbij worden de onderliggende biologische processen en de verschillende methoden die onderzocht zijn voor de preventie van nastaar uitgelegd en bediscussieerd.

In **hoofdstuk 3** wordt de relatie tussen de ontwikkeling van nastaar en de toename van stijfheid van de lens gedemonstreerd. We hebben een stress-relaxatie experiment uitgevoerd om de stijfheid van lenzen gevuld met siliconen vast te kunnen stellen. Het resultaat van dit experiment laat zien dat de lensstijfheid toeneemt met de ontwikkeling van nastaar. Dat is een belangrijke uitkomst, omdat de toename van lensstijfheid zorgt voor een afname van het accommoderend vermogen van de lens en dit heeft nadelige gevolgen voor het functioneren van injecteerbare accommoderende kunstlenzen. Mede daarom is de preventie van nastaar belangrijk voor de ontwikkeling van nieuwe technieken voor staaroperaties.

Flexibele en transparante siliconen polymeren zijn geschikt om een accommoderende kunstlens te vormen. **Hoofdstuk 4** beschrijft een studie waarin verschillende typen siliconen polymeren zijn getest. In onze studie wilden we kijken naar de invloed van siliconen op de lensstijfheid en naar de reactie van de lensepitheel cellen wanneer deze siliconen werden geïnjecteerd in het lenskapsel. De siliconen polymeren zijn gekozen uit de twee uiteinden van een spectrum: namelijk een hydrofobe silicone (ter-polymeer) en een hydrofiele silicone (PEG-gemodificeerde PDMS). We hebben geen relevante

verschillen gevonden in de vorming van nastaar bij lenzen gevuld met de verschillende siliconen. Dit geeft aan dat hydrofiliciteit als materiaal eigenschap niet relevant lijkt te zijn voor toekomstige toepassingen van siliconen voor injecteerbare accommoderende lenzen.

Onze studie in **hoofdstuk 5** werd uitgevoerd om het effect van hyaluronan (natrium hyaluronaat) op nastaar te onderzoeken. Hyaluronan is een component van de visco-elastica die tijdens staaroperaties worden gebruikt. Wij hebben aangetoond dat de reactie van de lensepitheel cellen kan worden beïnvloed door verschillende samenstellingen van hyaluronan die zijn opgelost in fysiologische zoutoplossing of gedemineraliseerd water. Toevoeging van het cytostaticum actinomycine D aan hyaluronan leidde tot volledige afwezigheid van lensepitheel cellen op het lenskapsel. Hoewel actinomycine D voorzichtig in de lens moet worden gebruikt wegens het risico op bijwerkingen voor andere weefsels in het oog hebben we met deze studie wel laten zien dat een combinatie behandeling met hyaluronan en actinomycine D gunstig is voor de preventie van nastaar.

In **hoofdstuk 6** laten we zien dat hydrogelen op basis van nanovezels (nanogelen) de lensepitheel cellen beïnvloeden. In deze studie hebben we de resultaten van behandeling met 14 verschillende combinaties van nanogelen met aangehechte peptiden (korte eiwitten) vergeleken. Deze combinaties zijn gemaakt met behulp van vijf peptiden die afkomstig zijn uit de extracellulaire matrix (ECM) componenten laminine, fibronectine en collageen IV. Deze ECM componenten zijn allen gerelateerd aan de ontwikkeling van nastaar door middel van de integrine receptoren in de lenscellen. Met deze studie hebben we bewijs geleverd voor de betrokkenheid van integrine receptoren bij het ontstaan van nastaar. Daarnaast hebben we met deze peptiden de reactie van de lensepitheel cellen kunnen beïnvloeden, wat resulteerde in een afname van de vorming van nastaar. Dit laat zien dat nanogelen veelbelovende materialen zijn voor onderzoek naar de preventie van nastaar. Deze resultaten zijn verder bekeken in het onderzoek dat in **hoofdstuk 7** is opgenomen, waar de verhoudingen van de peptiden in de nanogelen zijn geoptimaliseerd. Deze studie laat zien dat kleine veranderingen in de peptidenverhouding kunnen leiden tot grote effecten in de reactie van de lensepitheel cellen. Dit betekent dat de concentraties van de peptiden erg belangrijk zijn voor de signalen die in de integrine receptoren ontstaan. Verder geeft dit aan dat vervolgstudies nodig zijn om de verhoudingen tussen de peptiden te verbeteren om zo uiteindelijk te kunnen zorgen voor de meest optimale preventie van nastaar.

De experimenten in **hoofdstuk 8** zijn uitgevoerd om de methoden voor het bewaren van varkensogen in een wet lab te verbeteren. Een wet lab is een laboratorium waar chirurgische vaardigheden getraind kunnen worden met model-ogen of met dierlijk restmateriaal. Hiervoor worden veelal varkensogen gebruikt. Om goed te kunnen trainen is het van belang dat de hoornvliezen van deze ogen zo lang mogelijk transparant blijven en daarom hebben wij 15 verschillende bewaarmethoden getest. Het bewaren van de ogen in kraanwater zorgde voor de minste zwelling van de hoornvliezen en dat is gecorreleerd met een grotere transparantie van de hoornvliezen. Dit zijn interessante resultaten aangezien kraanwater goedkoop en universeel beschikbaar is. Deze resultaten kunnen er daarom voor zorgen dat het plannen van wet lab trainingen en experimenten gemakkelijker wordt.

Naast faciliteiten in een wet lab is chirurgische training ook belangrijk voor het ontwikkelen van nieuwe chirurgische technieken. In **hoofdstuk 9** evalueren we het belang van stereoscopisch zien voor chirurgische prestaties met de operatie microscoop. Stereoscopisch zien zorgt voor betere prestaties bij taken die worden uitgevoerd onder de operatiemicroscoop of met een staaroperatie simulator. Er zijn echter nog veel meer factoren van belang die de chirurgische vaardigheden kunnen beïnvloeden en weten we nog niet of de afwezigheid van stereoscopisch zien daadwerkelijk leidt tot een onvermogen om chirurgische taken naar behoren uit te voeren.

De uiteindelijke bevindingen van onze studies worden bediscussieerd in **hoofdstuk 10**. In dit hoofdstuk worden de resultaten vergeleken en worden er ideeën gegeven voor vervolgstudies. We denken dat nanomaterialen potentieel geschikt zijn voor nastaar preventie, aangezien nanomaterialen de biologische processen kunnen beïnvloeden die ten grondslag liggen aan de vorming van nastaar. Verder denken we dat een combinatie van meerdere technieken nodig zal zijn om nastaar te voorkomen.

De studies in dit proefschrift dragen bij aan de kennis van de onderliggende biologische processen van nastaarvorming. Er worden inzichten gegeven in mogelijke methoden voor nastaar preventie en er worden mogelijkheden gegeven om de chirurgische technieken van staaroperaties te verbeteren. Vervolgstudies zijn nodig om de specifieke processen die nastaar veroorzaken te verklaren. De uitdaging voor toekomstige onderzoeken is om biologisch gerichte interventies te ontwikkelen die uiteindelijk nastaar kunnen tegengaan.

Appendices

Dankwoord

Allereerst wil ik u bedanken voor het lezen van mijn proefschrift. Ik hoop dat u het allemaal een beetje heeft kunnen begrijpen.

Nu ik ben aangekomen bij het schrijven van het allerlaatste hoofdstuk van mijn proefschrift heb ik een dubbel gevoel. Enerzijds ben ik erg blij dat het proefschrift nu echt bijna af is, maar daarnaast staat het schrijven van het dankwoord ook voor de afsluiting van een mooie tijd die ik heb gehad als onderzoeker. Toen ik als student begon aan mijn wetenschappelijke stage had ik nooit verwacht dat ik daar zo enthousiast van zou worden dat ik daarna aan een promotieonderzoek van vier jaar zou beginnen. Terugkijkend is deze tijd omgevlogen en heb ik ontzettend veel geleerd wat ik ook zal meenemen in de kliniek. Daarom wil ik iedereen bedanken die mij heeft geholpen met de totstandkoming van dit proefschrift.

Een aantal mensen wil ik graag in het bijzonder bedanken, omdat ze een speciale rol hebben gehad:

Ten eerste wil ik mijn promotor en copromotoren bedanken voor hun steun en adviezen. Professor Hooymans, beste Anneke, ontzettend bedankt voor je hulp bij het bewaken van de planning gedurende de vier jaren van mijn promotieonderzoek en voor de goede tips de je mij hebt gegeven. Dr. Koopmans, beste Steven, jou wil ik bedanken voor je kritische vragen over het onderzoek en je hulp (en geduld) bij het aanpassen van de manuscripten. Daarnaast ben ik erg blij dat ik tijdens mijn promotieonderzoek ook in het wet lab heb kunnen opereren op varkensogen. Steven, bedankt dat je mij deze kans hebt gegeven en dat je de tijd hebt genomen om mij de chirurgische technieken te leren. Dr. van Kooten, beste Theo, van jou heb ik geleerd hoe leuk het werken op een lab kan zijn en welke mooie technieken en mogelijkheden er allemaal zijn om cellen te onderzoeken. Bedankt voor je hulp bij het onderzoek en op het lab bij de afdeling Biomaterialen.

De beoordelingscommissie, professor Luyten, professor Nuijts en professor Ren, wil ik bedanken voor de tijd die jullie hebben gemaakt om mijn proefschrift te lezen en te beoordelen.

De collega's van het EFRO project, Steven, Theo, Roel en Raquel, wil ik graag bedanken voor de nuttige bijeenkomsten en de tips voor de analyses van de materialen. Ook wil ik de collega's bij Nano-FM, Menno, Edith, Annemarie en Erna, bedanken voor het maken van de nanogel voor onze studies en voor jullie hulp bij de interpretatie van de data. Ik wil Edith in het bijzonder bedanken voor alle adviezen en steun tijdens het project: ook nadat je door een ongelukkig ongeval een periode uit de running bent geweest heb je toch veel moeite gedaan om ervoor te zorgen dat het project kon doorlopen en mij waar mogelijk geholpen. Verder wil ik Prashant van de afdeling Biomaterialen bedanken voor de hulp bij het uitvoeren van de viscoelasticiteits experimenten en Henk en Patricia van AMO bedanken voor hun betrokkenheid bij het project.

Ik heb mij naast het EFRO project ook bezig gehouden met neven projecten en daarvoor wil ik graag Thom bedanken voor de goede samenwerking en de leuke gesprekken. Frans wil ik bedanken voor de adviezen en hulp bij het opzetten van de stereoscopie studie.

Natuurlijk wil ik ook al mijn collega's van het LEO bedanken en dat waren er veel door de jaren heen: Else, Michael, Christiaan, Margriet, Francisco, Tim, Wietse, Esther, Bart, Shao Chong, Lisette, Nancy, Ruth, Doety, Bernadette en Ronald. Met jullie was het erg gezellig in het Laboratorium voor Experimentele Oogheelkunde (wat overigens geen écht lab is maar een saaie kantoortuin met gelukkig gezellige collega's). Bedankt voor jullie tips tijdens mijn onderzoek, de adviezen bij de Engelse taal, het beantwoorden van statistiek vragen, de tips voor goede restaurantjes en lekkere gerechten, de klusadviezen, maar vooral bedankt voor alle leuke gesprekken! Ook alle studenten die op het LEO onderzoek hebben gedaan wil ik bedanken voor de gezellige tijd: Wouter, Lukas, Marieke, Inge, Esra, Wilma, Simone, Marije en Marleen.

Zonder hulp van het secretariaat zou mijn onderzoek veel meer tijd hebben gekost. Ella en Stella, bedankt voor jullie assistentie bij het regelen van alle praktische zaken. Fenna, bedankt voor het eindeloos opzoeken van artikelen, voor je interesse in mijn onderzoek en je luisterend oor. Verder wil ik ook Joke, Wim, Wouter, Wim en Luuk bedanken voor alle hulp tijdens mijn promotieonderzoek.

Ik heb de afgelopen jaren ook veel tijd doorgebracht in het Skills Center en bij de afdeling Biomaterialen. Ik wil Hepke, Ingrid, Freddie, Camiel en Sip van het Skills Center

bedanken voor de hulp bij het regelen van alle praktische zaken voor het wet lab. Hepke, bedankt voor je assistentie bij het steriliseren van mijn instrumenten en voor je tips voor het verbeteren van de steriliteit bij mijn experimenten in het wet lab. Ik wil ook graag een aantal collega's van de Biomaterialen bedanken voor hun assistentie en gezelligheid op het lab: Corien, Hilde, Anna (Chongxia), Arina, Shao Chong, Raquel, Simon, Babs, Willem, Prashant, Willy en Ina.

De varkensogen voor mijn experimenten haalde ik 's ochtends bij Kroon Vlees op het Euvelgunne industrieterrein in Groningen. Zo begon ik altijd uitgewaaid mijn dag als ik bezig ging met mijn experimenten. Ik wil Rieks en zijn medewerkers bedanken voor de snelle verwerking van mijn bestellingen en voor het meedenken met mijn planning. Rieks wil ik in het bijzonder bedanken voor je vernieuwende kijk op de zaken, de leuke gesprekken tijdens de thee en je interesse in mijn onderzoek.

Ik wil mijn mentoren van de GSMS project management cursus, professor Rots en professor Faber, bedanken voor de goede tips, het luisterend oor en het overbrengen van het enthousiasme voor de wetenschap tijdens de sessies van de management cursus.

De editors van het W.J. Kolff News, Prashant, Hilde, Willy, Jenny (Ni), Willy, Patrick, Simon, Marieke en Yijin wil ik bedanken voor de leuke bijeenkomsten en goede samenwerking.

De organisatie van de DOPS 2015 conference meeting – An Eye for Music, Stanley, Maartje en Ronald, wil ik bedanken voor de gezellige bijeenkomsten en de geslaagde conferentie. Het is ons gelukt om oogheelkunde en muziek een beetje dichter bij elkaar te brengen.

Mijn paranimfen, Simone en Bart, wil ik natuurlijk bedanken voor het regelen van alle praktische zaken rondom mijn promotie, maar ook voor jullie interesse in mijn onderzoek. Simone, ik vind het ontzettend leuk dat je naast mijn zusje ook mijn collega bent geworden en ingeburgerd bent bij de afdeling Oogheelkunde. Voor mijn onderzoek ben je bezig geweest met celtellingen van microscopie plaatjes en dat heb je altijd met de grootste precisie gedaan, ondanks dat het niet het meest enerverende werk was. We zien elkaar elke week sowieso bij de orkestrepetitie, maar ik vond het ook erg leuk om op het werk gezellig samen met jou te gaan lunchen. Bart, je bent de enige collega

van de Oogheelkunde die ook het lab van de Biomaterialen kent en weet wat de ins en outs van het labonderzoek zijn. Daarom was het erg fijn om met jou te kunnen sparren over de uitdagingen waar we allebei wel eens tegenaan liepen. Jouw grote kennis van de celbiologie en ervaring in het labonderzoek hebben mij erg geholpen. Hoewel ik je (mannen-) grappen niet altijd begrijp, zorgt jouw aanwezigheid altijd voor leven in de brouwerij.

Ik wil mijn vrienden, familie en schoonfamilie bedanken voor jullie belangstelling in mijn onderzoek en voor alle gezellige momenten. De laatste periode voor het afronden van mijn proefschrift heb ik minder tijd voor jullie kunnen maken en daar hebben jullie altijd begrip voor gehad, bedankt daarvoor. Ondanks dat het soms lastig was om goed uit te leggen waar mijn promotieonderzoek over gaat zijn jullie altijd geïnteresseerd in waar ik mee bezig ben. Ik hoop dat jullie toch een deel van mijn proefschrift hebben kunnen lezen en begrijpen.

Lieve ouders, jullie wil ik bedanken voor alle steun door de jaren heen. Jullie hebben mij altijd gestimuleerd om het beste uit mijzelf te halen en mij onvoorwaardelijk gesteund in de keuzes die ik heb gemaakt. Ook nadat ik op kamers ging om geneeskunde te gaan studeren in Groningen stonden jullie altijd voor mij klaar. Alle keren dat ik ben verhuisd hebben jullie mij geholpen met verhuizen en met het opknappen van de kamers. Ook nu tijdens de verbouwing staan jullie altijd klaar om te helpen en komen jullie regelmatig naar Paterswolde om gezellig samen bezig te zijn.

Lieve Jan Willem, jij bent heel bijzonder voor mij. Zonder je onvoorwaardelijke steun en liefde had ik hier vandaag niet gestaan. Ik ben ontzettend dankbaar dat jij mij steunt in mijn enthousiasme voor mijn promotieonderzoek en voor de oogheelkunde in het algemeen. Ik vind het heel bijzonder dat je zoveel moeite wilt doen om je in te leven in mijn onderzoek en in de oogheelkunde, zodat wij hier samen over kunnen discussiëren. Daarnaast geef je mij de rust om te genieten van de natuur en van andere belangrijke zaken in het leven. Dankjewel.

Lisanne Nibourg
Paterswolde, november 2015

List of abbreviations

AC	Anterior chamber
ANOVA	Analyses of variance
αSMA	Alpha smooth muscle actin
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CAPE	Caffeic acid phenethyl ester
CCC	Curvilinear continuous capsulorhexis
CCT	Central corneal thickness
CDVA	Corrected distance visual acuity
CO	Capsular opacification
Co-Smad	Common-mediator Smad
COX-2	Cyclooxygenase-2
CsA	Cyclosporin A
CTGF	Connective tissue growth factor
DNA	Deoxyribonucleic acid
DGEA	Aspartic acid-glycine-glutamic acid-alanine, collagen IV-derived peptide
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transformation
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
HDAC	Histone deacetylase
HGF	Hepatocyte growth factor
ICAM	Intracellular adhesion molecule
IGF	Insulin-like growth factor
IKVAV	Isoleucine-lysine-valine-alanine-valine, laminin-derived peptide
ILK	Integrin linked kinase
IOL	Intraocular lens
I-Smad	Inhibitory or antagonistic Smad
LEC(s)	Lens epithelial cell(s)

LLCT	Low load compression tester
MAPK	Mitogen-activated protein kinase
MeCP2	Methyl CpG binding protein 2
MEM	Minimal essential medium
MMP	Matrix metalloproteinase
Nd:YAG	Neodymium-doped yttrium aluminum garnet
NMR	Nuclear magnetic resonance
NSAIDs	Non-steroidal anti-inflammatory drugs
PBS	Phosphate buffered saline
PBSA	Phosphate buffered saline containing 1% bovine serum albumin
PCO	Posterior capsular opacification
PDGF	Platelet-derived growth factor
PHSRN	Proline-histidine-serine-arginine-asparagine, fibronectin-derived peptide
PI3K	Phosphatidylinositol 3-kinase
RGDS	Arginine-glycine-aspartic acid-serine, fibronectin-derived peptide
RNA	Ribonucleic acid
R-Smad	Receptor-activated Smad
siRNA	Small interference RNA (ribonucleic acid)
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
US	Ultrasound
Wnt	Wingless-related integration site
YIGSR	Tyrosine-isoleucine-glycine-serine-arginine, laminin-derived peptide
5-FU	5-Fluorouracil

Bibliography

Nibourg L.M., Gelens E., Kuijer R., Hooymans J.M.M., van Kooten T.G., Koopmans S.A., 2015. Prevention of posterior capsular opacification. Experimental Eye Research. 136, 100-115.

Nibourg L.M., Sharma P.K., van Kooten T.G., Koopmans S.A., 2015. Changes in lens stiffness due to capsular opacification in accommodative lens refilling. Experimental Eye Research 134, 148-154.

Nibourg L.M., van Kooten T.G., Terwee T., Koopmans S.A., 2015. Effects of hyaluronan and sustained release of actinomycin D on capsular opacification after cataract surgery in a porcine eye model. *Submitted.*

Nibourg L.M., Gelens E., de Jong M., Kuijer R., van Kooten T.G., Koopmans S.A., 2015. Nano-fiber based hydrogels with extracellular matrix-based synthetic peptides for the prevention of capsular opacification. Experimental Eye Research, Epub.
Nibourg L.M., Gelens E., Nibourg S.A.F., de Jong M.R., Kuijer R., van Kooten T.G., Koopmans S.A., 2015. Effects of peptide ratios in nanofiber-based hydrogels for the prevention of capsular opacification. *Submitted.*

Nibourg L.M., Koopmans S.A., 2014. Preservation of enucleated porcine eyes for use in a wet laboratory. Journal of Cataract and Refractive Surgery. 40, 644-651.

Nibourg L.M., Wanders W., Cornelissen F.W., Koopmans S.A., 2015. Influence of stereoscopic vision on task performance with the operating microscope. Journal of Cataract and Refractive Surgery, Epub.

Curriculum Vitae

Lisanne Nibourg was born on the 29th of January 1987 and raised in Borne, a village in the eastern part of the Netherlands. After finishing her secondary school in 2005 at the Scholengemeenschap Twickel in Hengelo, she moved to Groningen to study Medicine at the University of Groningen. During this study she was active in several committees at the student faculty association and board member of the Groningen Talent Group. In 2009 she started her internships which were mainly followed at the University Medical Center Groningen and the Deventer Hospital. She did her final internship and research project at the ophthalmology department of the University Medical Center Groningen. For the research project she studied the importance of stereoscopic vision for task performance with the operating microscope under supervision of S.A. Koopmans, MD, PhD and F.W. Cornelissen, PhD. She graduated in August 2011 as a Medical Doctor and started her work as a PhD candidate in September 2011 at the department of ophthalmology of the University Medical Center Groningen under supervision of S.A. Koopmans, MD, PhD, T.G. van Kooten, PhD and Prof. J.M.M. Hooymans, MD, PhD on prevention of posterior capsular opacification. Since September 2015 she is a resident in training for ophthalmologist under the supervision of J.W.R. Pott, MD, PhD and Prof. N.M. Jansonius, MD, PhD at the University Medical Center Groningen.